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DNA/Dendrimer-Based Films

A novel material with potential biomedical applications

DOCTORAL THESIS

Rita Maria de Castro

DOCTORATE IN CHEMISTRY
SPECIALTY IN MATERIALS CHEMISTRY



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To my parents (Sidónio & Conceição),
my sister (Fernanda) and
my dog (Pituko)

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RESUMO

Nesta tese de doutoramento, foram desenvolvidos novos materiais híbridos, sob a forma de filmes finos, essencialmente baseados em interações eletrostáticas estabelecidas entre moléculas de ácido desoxirribonucleico (ADN) e dendrímeros de poli(amidoamina) (PAMAM). Os dendrímeros PAMAM são moléculas à escala nanométrica que apresentam multi-valência e um baixo índice de polidispersão. Esta classe de moléculas tem sido muito estudada para a entrega de genes e fármacos em células, para além de muitas outras aplicações na área biomédica. Isto apenas é possível devido às suas propriedades físicas, químicas e estruturais, que permitem a interação eletrostática com ácidos nucleicos e conjugação à superfície e/ou encapsulamento de fármacos no seu interior. Neste trabalho, foi tirado proveito da capacidade destas moléculas interagirem com o ADN para obter novos materiais com aplicações biomédicas promissoras.

ESTRUTURA DA TESE

O capítulo I fornece uma visão global do estado da arte relativamente a hidrogéis e filmes à base de ADN, onde o ADN é o componente essencial ou um componente responsável por conferir, a estes materiais, propriedades importantes e únicas. É dada atenção aos diferentes métodos utilizados para a sua obtenção, sendo também salientadas algumas das suas propriedades e aplicações práticas.

Os hidrogéis à base de ADN são materiais reticulados com capacidade de reter grande quantidade de água. Estes materiais podem ser categorizados com base na sua natureza química (pura ou híbrida), no tipo de interações estabelecidas entre os seus componentes (físicas ou químicas) ou no seu comportamento relativamente à aplicação de estímulos externos. As utilizações mais exploradas destes hidrogéis incluem a remoção de poluentes, os biosensores e reatores, os agentes de entrega de genes e fármacos, as matrizes tridimensionais para a cultura de células e as plataformas para a produção de proteínas *ex-vivo*.

No caso dos filmes à base de ADN, fez-se uma categorização de acordo com as metodologias mais frequentemente utilizadas para a sua preparação, nomeadamente pelas técnicas de Langmuir-Blodgett (LB), funcionalização covalente de superfícies (incluindo monocamadas auto-montadas (SAMs)), camada sob camada (LbL), de molde ("cast coating") e precipitação em banho químico ("wet spinning"). Destas, resultam diferentes tipos de filmes (monocamadas ou multicamadas), com distintas disposições moleculares (altamente organizadas ou aleatórias). Os filmes com elevado grau de organização têm sido utilizados para a compreensão e avaliação da estrutura do ADN sob

diferentes condições experimentais e na presença de diversos ligandos, sendo de extrema importância na área dos biosensores. Por outro lado, os filmes com um menor grau de organização (como os obtidos pela técnica de LbL, *via* interações eletrostáticas) têm sido aplicados para fins como a entrega de fármacos/genes.

O capítulo II inicia-se com uma breve introdução acerca dos dendrímeros PAMAM utilizados neste trabalho, nomeadamente a sua síntese, propriedades e aplicações. Em seguida, é reportado um método para a produção de novos materiais (filmes insolúveis em água) baseados em ADN e dendrímeros PAMAM. Primeiro, o ADN é aquecido acima da sua temperatura de desnaturação (para se obterem cadeias simples) e, em seguida, é misturado com os dendrímeros. Os parâmetros experimentais necessários para a formação do filme foram otimizados e foi estabelecido um protocolo geral para o processo. Também se ensaiou a preparação de filmes com outros polímeros para além dos dendrímeros PAMAM. A obtenção destes filmes é dependente da temperatura de aquecimento, da velocidade de arrefecimento, do pH inicial, da composição da solução, do tamanho/forma do ADN, bem como da geração dos dendrímeros e da razão N/P (aminas/fosfatos). A presença de ADN e dendrímeros PAMAM nestes filmes foi demonstrada, realizando-se também testes de estabilidade com estes materiais em condições fisiológicas, os quais revelaram uma grande estabilidade por longos períodos de tempo. A análise por microscopia eletrónica de varrimento mostrou uma superfície onde eram visíveis esferas interligadas. O potencial zeta da superfície deu positivo e as experiências de dicroísmo circular revelaram evidências de quiralidade supramolecular. Embora os filmes produzidos não tenham promovido a adesão celular, foi possível cultivar células na sua presença. A adesão celular pareceu melhorar após modificação dos filmes com péptidos arginina-glicina-ácido aspártico cíclicos, conhecidos por interagirem especificamente com as integrinas à superfície das células. Finalmente, os filmes foram testados como veículos para a entrega de fármacos anticancerígenos, nomeadamente o 5-fluorouracilo, a cisplatina e a doxorubicina. Os resultados mostraram que estes filmes apenas constituíam plataformas interessantes para a entrega de doxorubicina, a qual é um fármaco intercalador de ADN.

ABSTRACT

In this Ph.D. thesis, novel hybrid materials, in the form of films, were prepared, essentially based on electrostatic interactions established between deoxyribonucleic acid (DNA) molecules and poly(amidoamine) (PAMAM) dendrimers. PAMAM dendrimers are nanoscale molecules that show multi-valency and a low degree of polydispersion. These molecules have been widely studied as gene and drug delivery systems, among other biomedical applications. This is only possible due to their physical, chemical and structural properties, which allow for the electrostatic interaction with nucleic acids, surface conjugation and/or encapsulation of drugs. This work took advantage of the easy interaction of dendrimers with DNA to obtain a new material with promising biomedical applications.

THESIS CONTENT

Chapter I gives a general picture of the state of the art in DNA-based hydrogels and films, in which DNA is the main component and/or a component responsible for endowing these materials with important and unique properties. Attention is given to the different methods used to obtain such materials, as well as to their properties and practical applications.

DNA-based hydrogels are crosslinked materials that present a high water retention capacity. They may be generally categorized based on their chemical nature (pure or hybrid), the type of interactions established among their components (physical or chemical) or their responsiveness behaviour upon external stimuli. The most explored applications of these hydrogels include environmental pollutant removal, biosensors and reactors, gene and drug delivery, tridimensional scaffolds for cell culture, and platforms for cell-free protein synthesis.

The classification of DNA-based films is made according to their most common preparation methodologies, namely, the Langmuir-Blodgett (LB) technique, covalent surface functionalisation (including Self-Assembled Monolayers (SAMs)), Layer-by-Layer self-assembly (LbL), solution casting techniques and wet-spinning methods. Using these approaches, different types of films may be obtained (monolayers and multilayers), with distinct molecular arrangements (highly organized or random oriented). Films with a high organization degree have been used to understand and evaluate the DNA structure under different experimental conditions and in the presence of several ligands, being mainly applied in biosensing. On the other hand, films with lower organization degree (such as electrostatically driven LbL) have been used for applications such as drug/gene delivery.

Chapter II starts by a brief introduction focused on the PAMAM dendrimers used in this work, namely their synthesis, properties and applications. Next, a methodology to produce novel materials (water-insoluble films) based on DNA and PAMAM dendrimers is reported. First, the temperature of DNA is increased above its melting temperature (T_m), in order to obtain single stranded (ssDNA), and, at that point, dendrimers are introduced in the system. The experimental parameters required for film formation were optimized and a general protocol for film preparation was established. Beyond PAMAM dendrimers, other polymers were also assayed for film production. The formation of these new DNA/PAMAM dendrimer films was dependent on the DNA heating temperature, cooling speed, initial pH, buffer composition, DNA size/shape, dendrimer generation and N/P (amine/phosphate) ratio. The presence in the films of DNA and PAMAM dendrimers was demonstrated and studies under physiological conditions revealed that these materials were highly stable over long time periods. The analysis of the films by scanning electron microscopy (SEM) showed a homogeneous surface that seemed to contain interconnected spheres. Furthermore, zeta potential measurements conducted to positive values and circular dichroism analysis revealed evidences of supramolecular chirality. Although the produced films did not promote cell adhesion, it was possible to culture cells in their presence. Also, cell adhesion appeared to improve by modification of the films with cyclic arginine-glycine-aspartic acid peptides, known for their specific interaction with integrins existent at the cell membrane. Finally, the films were tested as vehicles for the delivery of anticancer drugs, namely 5-fluorouracil, cisplatin and doxorubicin. The results showed that these films were only interesting platforms for the delivery of doxorubicin, which is a DNA intercalating drug.

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ACRONYMS

A	Adenine	DSC	Differential scanning calorimetry
Aa	9-aminoacridine hydrochloride	dsDNA	Doubled stranded DNA
AA	Antibiotic-antimycotic	DTT	Dithiothreitol
ABOL	4-aminobutanol	EB or EtBr	Ethidium bromide
AET	2-aminoethanethiol	EC₅₀	Effective concentration 50%
AgNP	Silver nanoparticles	ECM	Extracellular matrix
Al₂(C₄BP)	Aluminum (III) alkanebisphosphonate	EDA	Ethylenediamine
ALG	Alginate	EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
AO	Acridine orange	EDTA	Ethylenediaminetetraacetic acid
ASO	Antisense oligonucleotide	EGDE	Ethylene glycol diglycidyl ether
ASV	Anodic stripping voltammetry	EGFP	Enhanced green fluorescent protein
ATP	Adenosine triphosphate	EKA	ElectroKinetics Analyzer
AuNP	Gold nanoparticles	EMCS	6-maleimidohexanoic acid N- hydroxysuccinimide ester
BCA	Bicinchoninic acid	EPIR	Epirubicin
bp	Base pairs	FBS	Foetal bovine serum
br	Branched	FDA	Fluorescein diacetate
PEI	Poly(ethyleneimine)	FdUMP	2'-Deoxy-5-fluorouridine 5'- monophosphate
BSA	Bovine serum albumin	FdUTP	5-fluorodeoxyuridine triphosphate
C	Cytosine	FESEM	Field-emission scanning electron microscopy
CB[8]	Cucurbit[8]uril	FITC	Fluorescein isothiocyanate
CBA	Cystamine bisacrylamide	FUTP	Fluorouridine-5-triphosphate
CD	Circular dichroism	G	Guanine
CE	Crown ether	GA	Glutaraldehyde
CHI	Chitosan	GFAP	Glial fibrillary acidic protein
cisPt	Cisplatin	GNP	Gold nanoparticles
CLSM	Confocal laser scanning microscopy	GO	Graphene oxide
CMC	Carboxymethyl cellulose	GOx	Glucose oxidase
COL	Chitosan oligosaccharide lactate	GSH	Glutathione
CPT	Camptothecin	H33258	Hoechst33258
cRGD	Cyclic RGD	HA	Hyaluronic acid
CTAB	Cetyltrimethylammonium bromide	HAc	Glacial acetic acid
CTR	Controlled temperature ramp	HCR	Hybridization chain reaction
cys	Cysteamine	HIS	Histamine
D or Dx	Dextran	HRP	Horseradish peroxidase enzyme
DAB	1,4-diaminobutane	HT	Hexanethiol
DAPI	4',6-diamidino-2-phenylindole	IL	Ionic liquid
DDR	DNA displacement reaction	IPN	Interpenetrating network
DMEM	Dulbecco's Modified Eagle Medium	KP	Krytox
DMF	Dimethylformamide	LB	Langmuir-Blogett
DMSO	Dimethylsulfoxide	LS	Langmuir-Schaefer
DNA	Deoxyribonucleic acid	LE	Loading efficiency
DOTA	Tetrazetane	L-glut	L-glutamine
DOX or DXR	Doxorubicin	In	Linear

LS	Langmuir-Schaefer	PTC	Peltier type cell
MAP2	Microtubule-associated protein 2	PV	Phenylenevinylene
MCA	Multi-primed chain amplification	PVA	Poly(vinylalcohol)
MCH	6-mercapto-1-hexanol	PVD	Physical vapour deposition
MD	Molecular dynamics	PVS	Poly(vinylsiloxane)
MPA	Mercaptopropionic acid	QCM	Quartz crystal microbalance
MTT	3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide	QD	Quantum dots
MWCNT	Multiwalled carbon nanotubes	RCA	Rolling circle amplification
MWCO	Molecular weight cut-off	RITC	Rhodamine B isothiocyanate
N/P	Ratio between amine and phosphate	rpm	Rotation per minute
ODN	Oligonucleotide	RT	Room temperature
OPDA	<i>ortho</i> -phenylenediamine	RTIL	Room temperature ionic liquid
OVA	Ovalbumin	SAM	Self-assembled monolayers
PA	Poly(acrylamide)	SC	SuperClear microcentrifuge tube
PAA	Poly(acrylic acid)	SEI	Secondary electron image
PAAs	Poly(amidoamines)	SEM	Scanning electron microscopy
PAH	Poly(allylamine)	siRNA	Small interfering RNA
PAMAM	Poly(amidoamine) dendrimer	SP	Spermine
PARG	Poly(arginine)	SPR	Surface plasmon resonance
PBS	Phosphate buffered saline	SPS	Solution phase synthesis
PCL	Poly(caprolactone)	ssDNA	Single stranded DNA
PCR	Polymerase chain reaction	SS-DNA	Sodium salt DNA
PDDA	poly(diallyldimethylammonium)	Sulfo-NHS	N-hydroxysulfosuccinimide
pDMA-co-NAS	Poly(N,N-dimethylacrylamide-co-N-acryloyloxysuccinimide)	SWNT	Single walled nanotubes
PDMS	Poly(dimethylsiloxane)	T	Thymine
pDNA	Plasmid DNA	TA	Tannic acid
PEG	Poly(ethylene glycol)	TAT	Transactivator protein
PEI	Poly(ethyleneimine)	TBA	Thrombin-binding aptamer
PEM	Polyelectrolyte multilayers	TE	Tris-EDTA buffer
PEO	Poly(ethylene oxide)	TEM	Transmission electron microscopy
PEVAC	Poly(ethylene-co-vinyl acetate)	TEMED	Tetramethylethylenediamine
PLA	Poly(lactic acid)	Tf	Transferrin
pLGA-co-pLG	Poly(L-glutamic acid-co-γ-propargyl-L-glutamate)	Tm	Melting temperature
PLL	Poly(L-lisine)	TMPyP	5,10,15,20-tetrakis(4-N-methylpyridyl)porphine tetra(p-toluenesulfonate)
PMMA	Poly(methylmethacrylate)	TMR-D	Tetramethylrhodamine-dextran
pNIPAM	Poly(N-isopropylacrylamide)	TPA	Terephthalaldehyde
PPV	Poly(phenylenevinylene)	TS	Thymidylate synthase
PrS	Protamine sulfate	UV-vis	Ultraviolet-visible
PS	Poly(styrene)	ZP	Zeta-potential
PSS	Poly(4-styrenesulfonic acid)		

SHORT CURRICULUM VITAE

Rita Castro was born in 1988, in Kathu, South Africa. She graduated in Biochemistry in 2009 (15/20) and concluded the Master in Applied Biochemistry in 2011 (18/20), both at the *Universidade da Madeira* (UMa). In 2012, she was awarded a Ph.D. grant from the *Fundação para a Ciência e Tecnologia* (FCT) and, since then, she is a Ph.D. Student at UMa, developing the experimental work under the scope of the Molecular Materials Research Group (MMRG) at *Centro de Química da Madeira* (CQM). Her Ph.D. work was done under the supervision of Dr. Helena Tomás (CQM and UMa) and co-supervision of Dr. Ana Paula Pêgo (*Instituto de Investigação e Inovação em Saúde* - i3S, Porto). During the Ph.D. period, she participated in several advanced courses and scientific meetings. She has also contributed for several research projects and activities developed at CQM and UMa that are described in more detail below.

As a result of her scientific activity, she is author/co-author of 1 Thesis, 10 papers in international peer-reviewed journals (7 published and 3 still in preparation), 1 book chapter, and 1 abstract in a peer-reviewed journal. Also, her work resulted in 18 oral communications (4 international and 14 in CQM meetings) and 9 poster presentations (all international) in scientific meetings.

PARTICIPATION IN RESEARCH PROJECTS

Project DENDRIMAT – New materials for drug/gene delivery based on the self-assembly of dendrimer-chitosan-single stranded DNA (Ref. PTDC/CTM-NAN/112428/2009). Research grant (BI-Master) in the period January **2012** - September **2013**.

PARTICIPATION IN ADVANCED COURSES

- ✓ Gordon Research Center on Cancer Nanotechnology. Mount Snow in West Dover, VT, United States of America: June 18 - 23, **2017**.
- ✓ II Jornadas Nacionais de Caracterização de Materiais. Ciceco, University of Aveiro. Aveiro, Portugal: January 24 - 26, **2017**.
- ✓ Nanoschool 2016: "Principles and Applications of Fluorescence Spectroscopy" (8h, October **2016**). Lecturer: Prof. Fernando Lahoz, University of La Laguna (Spain).
- ✓ Nanoschool 2015: "Bionanotechnology: From Bio to Nano & From Nano to Bio" (8h, October **2015**). Lecturer: Prof. Hiroshi Matsui, City University of New York (USA).

- ✓ Nanoschool 2014: "Nanomedicines: Considerations for Design and Transfer into Clinical Use" (8h, October **2014**). Lecturer: Prof. Ruth Duncan, Cardiff University (UK).
- ✓ Nanoschool 2013: "Nanomedicines: the Next Generation of Pharmaceuticals" (8h, October **2013**). Lecturer: Prof. Serge Mignani, University of Paris Descartes (France).
- ✓ Nanoschool 2012: "Design, synthesis and biofunctionalization of magnetic nanoparticles: current and prospective *in vivo* applications" (8h, October **2012**). Lecturer: Prof. Etienne Duguet, University of Bordeaux (France).
- ✓ Nanoschool 2011: "From chemical topology to cages and molecular machines: the transition metal approach" (8h, October **2011**). Lecturer: Prof. Jean-Pierre Sauvage, University of Strasbourg (Nobel Prize in Chemistry 2016).
- ✓ Nanoschool 2010: "Inorganic nanoparticles: from synthesis to applications"(8h, October **2010**). Lecturer: Prof. Mathias Epple, University of Duisburg-Essen (Germany).
- ✓ Nanoschool 2009: "Nanochemistry and Supramolecular Chemistry" (8h, October **2009**). Lecturer: Prof. Kari Rissanen, University of Jyväskylä (Finland).
- ✓ Nanoschool 2008: "Photonic materials" (8h, October **2008**), Prof. Luisa de Cola, University of Münster (Germany).

OTHER COURSES

- ✓ "Introdução à Gestão de Resíduo Perigosos": 19 of January **2016** (University of Madeira).
- ✓ "Science Communication Short Course": 17 - 18 of April **2015** - Hernani Zão (CQM).
- ✓ Level 1 Competency on IZON's Tunable Resistive Pulse Sensing (TRPS) System: 2 of April **2013**. Certified by Dr. Rebecca Warr, IZON Science Limited.
- ✓ PTNMR - 2nd NMR Course – "Short Theoretical and Practical Introduction in the NMR Technique": 13 - 14 of December **2012** – João Figueira (CQM).
- ✓ 1st Course on "Fundamentals and Applications of Electrospinning Technology": 15 of June **2012** – Shili Xiao (CQM).
- ✓ PTNMR - 1st Edition of NMR Course - "Short theoretical and practical introduction in the NMR technique": 5 – 7 of December **2011** - Dan Domide (CQM).
- ✓ 2nd MS Course "Introduction to the Fundamentals of Mass Spectrometry and Basic Training on Electrospray Ion Trap Multistage Mass Spectrometer": 11 – 12 of November **2011** - Ying Lu (CQM).
- ✓ 1st MS Course "Basic Training on Operation of an Electrospray Ion Trap Multistage Mass Spectrometer": 21 - 22 February **2011** - Ying Lu (CQM).
- ✓ "LC-MS techniques: fundamentals and applications": 4 - 5 of November **2010** (CQM).

PUBLICATIONS

Master Thesis

Castro R. New Delivery Systems Based on Dendrimers with Applications in Antisense Therapy. Master Thesis. Funchal: Universidade da Madeira, **2011**.

Papers in international scientific periodicals with referees

1. Castro R, Alves CS, Granja P, Rodrigues J, Pêgo AP, Tomás H. DNA in materials science: hydrogels and films (in preparation).
2. Castro R, Vidal R, Silva F, Granja P, Rodrigues J, Pêgo AP, Tomás H. Hybrid DNA/Dendrimer Films with Supramolecular Chirality (in preparation).
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7. Nouri A, Castro R, Santos JL, Fernandes C, Rodrigues J, Tomás H. Calcium phosphate-mediated gene delivery using simulated body fluid (SBF). *Int J Pharmaceut* **2012**; 434:199-208.
8. Nouri A, Castro R, Kairys V, Santos JL, Rodrigues J, Li Y, Tomás H. Insight into the role of N,N-Dimethylaminoethyl methacrylate (DMAEMA) conjugation onto poly(ethylenimine): cell viability and gene transfection studies. *J Mater Sci: Mater Med* **2012**; 23:2967-2980.
9. Gonçalves M, Castro R, Rodrigues J, Tomás H. The Effect of PAMAM Dendrimers on Mesenchymal Stem Cell Viability and Differentiation. *Curr Med Chem* **2012**; 19:4969-4975.
10. Wang S, Castro R, Song C, Luo Y, Shen M, Tomás H, Zhu M, Shi X. Electrospun laponite-doped poly(lactic-co-glycolic acid) nanofibers for osteogenic differentiation of human mesenchymal stem cells. *J Mater Chem* **2012**; 22:23357–23367.

Book Chapters

Pêgo AP, Moreno P, Castro R, Tomás H. Nucleic Acid Delivery by Polysaccharide Based Vectors. In: Gil MH, editor. Carbohydrates Applications in Medicine. Research Signpost/Transworld Research Network (2014). ISBN: 978-81-308-0523-8.

Abstracts in international scientific periodicals with referees

Silva CL, Castro R, Perestrelo R, Tomás H, Câmara J. Effect of red wine bioactive polyphenols in Caco-2 cell line viability. *JISANH*. **2016**; DOI: 10.18143/JISANH_v3i4_1389.

ORAL COMMUNICATIONS IN INTERNATIONAL MEETINGS

1. Castro R, Granja P, Rodrigues J, Pêgo A, Tomás H. DNA/PAMAM Dendrimer Gel-Like Films as Potential Drug Delivery Platforms. Madeira International Conference - Emerging Trends and Future of Nanomaterials for Human Health (MAD-Nano16). Funchal, Portugal: November 17 - 20, **2016**.
2. Castro R, Granja P, Rodrigues J, Pêgo A P, Tomás H. Hybrid DNA/Dendrimer Films with Supramolecular Chirality. 5th Biodendrimer 2016. Copenhagen, Denmark: August 2 - 5, **2016**.
3. Olival A, Castro R, Rodrigues J, Tomás H. Endocytosis and Intracellular Trafficking of PEGylated PAMAM Dendrimers - Preliminary Studies. Indo-Portuguese Workshop on Emerging Trends of Nanotechnology in Chemistry and Biology (INCB-2016). New Delhi, India: February 12 - 13, **2016** (Invited Lecture).
4. Castro R, Santos J L, Gonçalves M, Rodrigues J, Tomás H. Antisense Therapy: Oligonucleotide Delivery Systems Based on Fatty-acid Functionalized PAMAM Dendrimers. 1st International Conference on Emerging Trends of Nanotechnology on Drug Discovery (INDD-2014). New Delhi, India: May 26 - 27, **2014**. (Invited Lecture)

ORAL COMMUNICATIONS IN CQM MEETINGS

1. Castro R, Granja P, Rodrigues J, Pêgo A, Tomás H. DNA/Polycation Hybrid Films: a Potential Doxorubicin Controlled Release Platform. 4th CQM Annual Meeting. Funchal, Portugal: February 3 - 4, **2017**.
2. Castro R, Granja P, Rodrigues J, Pêgo A P, Tomás H. Facile Synthesis of Hybrid DNA/Cationic Polymer Films. 3rd CQM Annual Meeting. Funchal, Portugal: April 1 - 2, **2016**.
3. Olival A, Castro R, Rodrigues J, Tomás H. PEGylated PAMAM Dendrimers: extent of Cell Uptake, Endocytosis Mechanism and Final Fate. 3rd CQM Annual Meeting. Funchal, Portugal: April 1 - 2, **2016**.
4. Zhao Y, Castro R, Zhu J, Tomás H, Shi X. PEGylated Polyethyleneimine-Entrapped Gold Nanoparticles for Enhanced and Targeted Gene Delivery Applications. 2nd CQM Annual Meeting. Funchal, Portugal: January 30 - 31, **2015**.
5. Castro R, Xiao S, Granja P L, Rodrigues J, Pêgo A P, Tomás H. Using DNA and PAMAM Dendrimers to Prepare a Film with Potential Drug/Gene Delivery Applications. 2nd CQM Annual Meeting. Funchal, Portugal: January 30 - 31, **2015**.

6. Sousa I J, Castro R, Rodrigues J, Tomás H. Synthesis of Chitosan-PAMAM Dendrimer Nanoparticles. 1st CQM Annual Meeting and 9th CQM Materials Group Meeting. Funchal, Portugal: January 31 - February 1, **2014**.
7. Castro R, Xiao S, Granja P L, Rodrigues J, Pêgo A P, Tomás H. New Materials Involving Self-Assembly of Dendrimer-Chitosan-Single Stranded DNA for Future Applications on Drug/Gene Delivery. 1st CQM Annual Meeting and 9th CQM Materials Group Meeting. Funchal, Portugal: January 31 - February 1, **2014**.
8. Castro R, Xiao S, Rodrigues J, Granja P L, Pêgo A P, Tomás H. First Studies in the scope of the project "DENDRIMAT-New Materials for Drug/Gene Delivery Based on the Self-Assembly of Dendrimer-Chitosan-ssDNA". 8th Materials Line Meeting. Funchal, Portugal: January 25, **2013**.
9. Gouveia M, Jardim M G, Castro R, Tomás H, Rodrigues J. Preparation of Low-Generation Metallodendrimers Using Nitrile-Functionalized Poly(alkylidenamines) Dendrimers: Cytotoxicity Studies in Cancer Cell Lines. 8th Materials Line Meeting. Funchal, Portugal: January 25, **2013**.
10. Alves CS, Gonçalves M, Castro R, Rodrigues J, Tomás H, Li Y. Dendrimer/Gold Nanoparticles for Gene Delivery. 8th Materials Line Meeting. Funchal, Portugal: January 25, **2013**.
11. Nouri A, Castro R, Santos J L, Fernandes C, Rodrigues J, Tomás H. Calcium-Phosphate Mediated Transfection Using Simulated Body Fluid (SBF). 7th Materials Line Meeting. Funchal, Portugal: January 27, **2012**.
12. Castro R, Rodrigues J, Tomás H. New Delivery Systems Based on Dendrimers with Applications in Antisense Therapy. 7th Materials Line Meeting. Funchal, Portugal: January 27, **2012**.
13. Gonçalves M, Castro R, Shi X, Rodrigues J, Tomás H. Evaluation of the Effect of Dendrimers with Different Surface Functionalities on the Differentiation of Mesenchymal Stem Cells. 6th Materials Line Meeting. Funchal, Portugal: January 28, **2011**.
14. Castro R, Rodrigues J, Tomás H. Surface Modified PAMAM Dendrimers in Antisense Therapy. 6th Materials Line Meeting. Funchal, Portugal: January 28, **2011**.

POSTER COMMUNICATIONS IN INTERNATIONAL SCIENTIFIC MEETINGS

1. Castro R, Granja P, Rodrigues R, Pêgo A, Tomás H. Hybrid DNA/polycation Films: A New System with Potential for Local Anticancer Therapy. Gordon Research Center on Cancer Nanotechnology. Mount Snow in West Dover, VT, United States of America: June 18 - 23, **2017**.
2. Castro R, Granja P, Rodrigues R, Pêgo A, Tomás H. Electrostatically Crosslinked DNA/Polycation Films: A Potential Doxorubicin Controlled Release Platform. II Jornadas Nacionais de Caracterização de Materiais. Aveiro, Portugal: January 24 - 26, **2017**.

3. Olival A, Castro R, Rodrigues J, Tomás H. Synthesis, Cytotoxicity and cellular uptake of PEGylated PAMAM Dendrimer. Indo-Portuguese Workshop on Emerging Trends of Nanotechnology in Chemistry and Biology (INCB2016). New Delhi, India: February 12 - 13, **2016**.
4. Castro R, Santos JL, Gonçalves M, Rodrigues R, Tomás H. Fatty-acid Functionalized PAMAM Dendrimers for Gene Silencing. 8th International Dendrimer Symposium. Madrid, Spain: September 5 - 8, **2013** (with flash presentation)
5. Gonçalves M, Castro R, Rodrigues J, Tomás H. PAMAM Dendrimers do not affect mesenchymal stem cells differentiation. 8th International Dendrimer Symposium. Madrid, Spain: June 23 - 27, **2013**.
6. Xiao S, Castro R, Rodrigues J, Tomás H. Novel PAMAM dendrimers/pDNA-coated magnetic iron oxide nanoparticles for theranostics. 8th International Dendrimer Symposium. Madrid, Spain: June 23 - 27, **2013**.
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8. Gonçalves M, Castro R, Rodrigues J, Tomás H. Effect of PAMAM Dendrimers with Different Surface Functionalities on Mesenchymal Stem Cells Differentiation. 7th International Meeting of the Portuguese Society for Stem Cells & Cell Therapies. Porto, Portugal: April 26 - 27, **2012**.
9. Nouri A, Li Y, Castro R, Santos JL, Rodrigues J, Tomás H. *In vitro* study of poly(ethylenimine) (PEI)-g-dimethylaminoethyl methacrylate (DMAEMA) as a gene delivery vector. 9th International Symposium on Frontiers in Biomedical polymers - FBPS. Funchal, Portugal: May 9 - 12, **2011**.

OTHER ACTIVITIES

- ✓ Member of the Organizing Committee of the 4th CQM Annual Meeting, Funchal, Madeira Island, Portugal (3 - 4 February **2017**).
- ✓ Participation in several science dissemination activities carried out in the scope of CQM, of which should be highlighted *A Química é Divertida* ("Chemistry is fun"). Collaborator in the period **2006 - 2016**.
- ✓ Member of the Organizing Committee of MAD-Nano16: Madeira International conference on Emerging Trends and Future of Nanomaterials for Human Health, Funchal, Madeira Island, Portugal (17 - 20 November **2016**).
- ✓ Collaboration in several laboratory lessons of the 1st Cycle in Biochemistry and 2nd Cycle in Applied Biochemistry offered by UMa. Collaborator in the period **2010 - 2017**.

Chapter I

DNA in Materials science: hydrogels and films

Abstract

Since the earliest times, deoxyribonucleic acid (DNA) has been the most important molecule in the world, as it contains all the genetic information needed for life existence. Even more than 60 years after Watson and Crick's greatest discovery and all the (bio)technological evolution, for sure, DNA still encloses some secrets that are yet to be discovered. But it will not be because of this that the research will stop, on the contrary, it will encourage "DNA diggers" to dig deeper until they reach the "gold mine". In the meanwhile, over the past 60 years, DNA has been widely explored in many scientific fields. This review will be focused on the latest research in the materials field using DNA as the key component, more precisely on DNA-based hydrogels and films. One will start with a brief insight of DNA's discovery and its most important properties, followed by evidences of its versatility in materials science. Then, the different chemical and physical approaches to obtain DNA hydrogels and films will be detailed, together with representative examples in the field. The stimuli-responsive properties of these materials and their practical applications will also be highlighted.

Keywords: DNA; DNA-based hydrogels; DNA-based films; stimuli-responsive materials; film deposition techniques

1. The DNA molecule and the world of science

Since the origin of life, DNA has played a pivotal role in passing on the genetic information encoded in the cell nucleus over generations (Figure 1, A). After water, DNA is considered to be one of the most important molecules in life and is thus often referred to as the "key to life"¹. Initially proposed to have an "inside out" triple helix nucleic acid structure by Pauling and Corey², Watson and Crick³ disproved this DNA model in the 1950's. Their discovery not only led to the first steps in unravelling the very complex process of DNA replication⁴, but it also allowed for the exploration of the potential genetic implications of DNA⁵.

The molecular structure of DNA proposed by Watson and Crick in 1953 was determined through X-ray diffraction analysis of a DNA fibre^{3,5}. The double helix structure of DNA (Figure 1, B) is composed of two 5'→3' single stranded DNA (ssDNA) chains, each made up of a long sequence of building blocks called nucleotides. Each nucleotide contains three distinct chemical sub-units: an external backbone composed of phosphate group (responsible for DNA's negative charge) and a five-carbon sugar (deoxyribose in the case of DNA) and an internal nitrogenous base. Four nitrogenous bases exist, namely adenine (A), guanine (G) (both purines), thymine (T) and cytosine (C) (both pyrimidines). Due to specific base complementarity, where A always pairs with T *via* a double hydrogen bond (A=T) and C pairs with G by a triple hydrogen bond (C≡G), the two ssDNA chains bind to each other in a complementary antiparallel manner (opposite directions). This leads to the formation of double stranded DNA (dsDNA), with the internal base pairs stacked on top of each other and the sugar-phosphate backbone on the outside of the structure. The dsDNA adopts a double helix conformation⁶⁻⁸, due to the "twist angle" and "propeller twist" properties of the base pairs⁹.

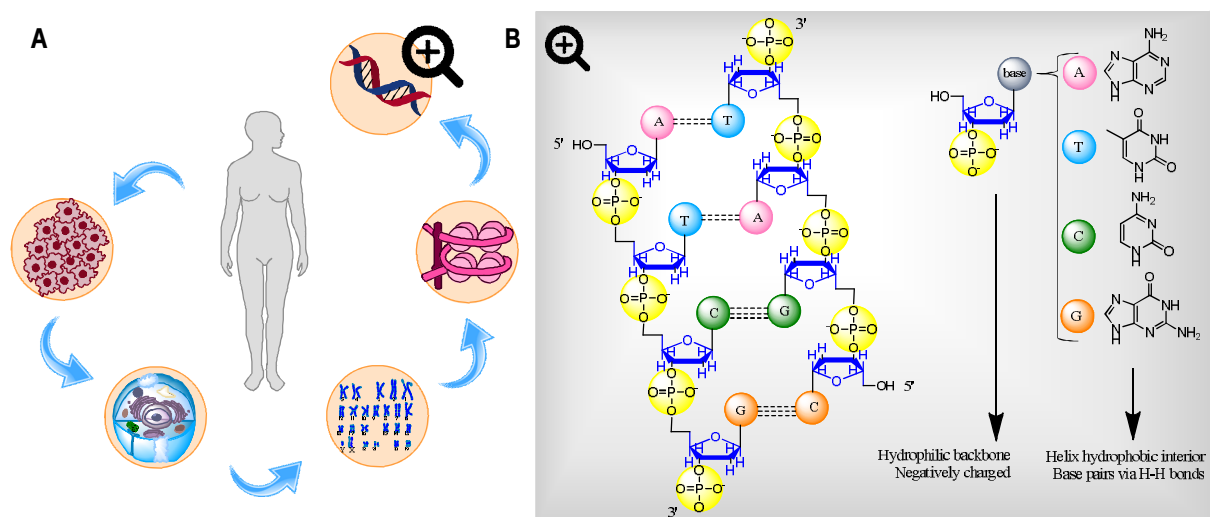


Figure 1 - DNA, the genetic information carrier and its basic unit, nucleotides. (A) DNA enclosed within the cell nucleus in eukaryotes in a very condensed form by histones. (B) Base pairs (AT and CG) in the DNA double helix.

According to the literature, three main DNA helix families are known to exist: A-, B- and Z-forms⁹. The B-form, which corresponds to the DNA conformation described by Watson and Crick³, is most common in living cells, while the Z-form is the rarest. In each case, helix formation comes from base stacking on top of each other, with slight twists, in order to maximize their hydrophobic interaction⁹. This creates a hydrophobic environment in the helix core that is surrounded by a hydrophilic sugar-backbone. The structure of each DNA helix family is known to vary dependent on hydration state, DNA sequence and the presence of proteins that can promote a conformational change¹⁰. The sequence-dependency makes it possible to find DNA sequences with regions in several conformations^{9,10}. Thus, different sequences of base pairs will result in different twisting angles, influencing the tightness, shape and orientation of the helices^{9,10}.

The DNA double helix is considered a stable structure, mainly due to the base stacking and the hydrogen bonds^{9,10}. Disruption of dsDNA into two strands is however possible through a process referred to as DNA denaturation. The reverse process is denominated hybridization. DNA denaturation can be achieved in two ways, either by enzymes⁴ (e.g. during the replication process) or by external chemical (e.g. denaturants) and physical (e.g. temperature) conditions¹¹⁻¹⁷. The most used method to achieve dsDNA denaturation is temperature increase. The temperature at which half of the DNA is denatured (*i.e.* where dsDNA and ssDNA coexist at a ratio of 1:1) is called the transition or melting temperature (T_m). This value falls in the range of 50°C to 100°C and depends on the DNA sequence, solvent and existence of solutes in solution¹⁸⁻²⁰. The evolution of dsDNA denaturation by temperature increase can easily be monitored, with the most commonly used method for this being UV-visible absorbance²¹. An important correlation exists between the T_m and the number and sequence of each base pair, where the nearest-neighbor interactions have been shown to play a significant role²². Generally, the T_m increases in a linear manner with the percentage of G-C base pairs (Figure 2, A)²¹. Relative to the A-T pair, the G-C pair has a higher T_m value indicating that it is more stable. Accounting for this is the triple hydrogen bond in G-C, as opposed to the double hydrogen bond maintained in A-T. When dsDNA is submitted to a temperature increase, a slight increase in absorbance right at the beginning of the transition occurs. As the temperature continues to increase, the hydrogen bonds between the two strands as well as the stacking interactions are disrupted and the two strands begin to separate. As the separation process continuous, the absorbance in the DNA sample continues to increase. The reason behind this is that the stacked purine and pyrimidine bases in the double helix absorb less ultraviolet light due to the stacking interactions²³. This phenomenon is known as hyperchromicity or a hyperchromic shift (Figure 2, B)²⁴. Other methods can be used to confirm dsDNA denaturation, namely, fluorescence measurements using ethidium bromide (EtBr) and dynamic rheological measurements^{23,25}.

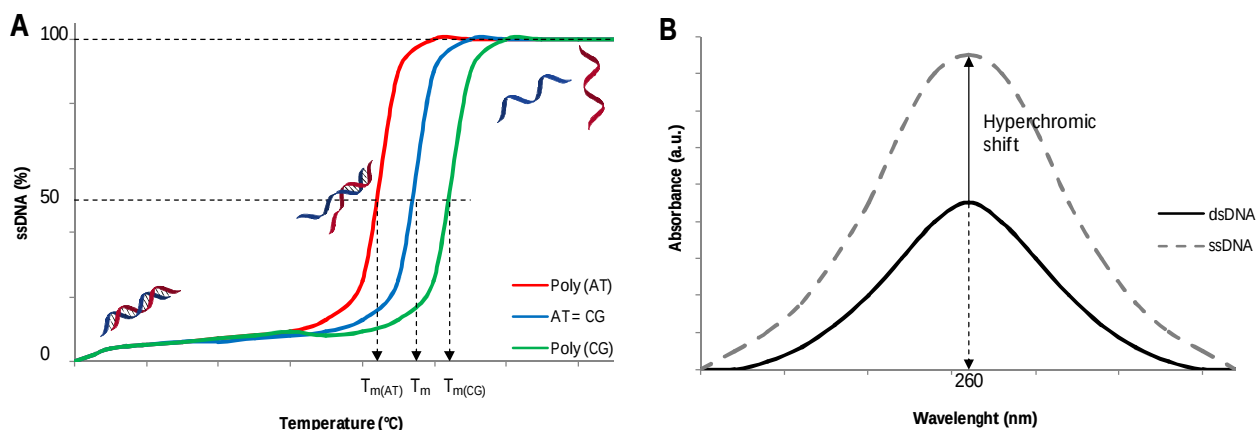


Figure 2 - DNA denaturation stages and absorbance changes. (A) dsDNA behaviour upon temperature increase and its sequence dependency. (B) Hyperchromic shift upon dsDNA denaturation into ssDNA, resulting in an increase in the absorbance at 260nm.

DNA is a biomacromolecule that can suffer random degradation (covalent bond disruption leading to breakage of DNA into smaller fragments) under specific conditions, such as alterations in pH and temperature, as well as exposure to chemical compounds, radiation, sonication, shearing and enzymes^{26–33}. It can also undergo precise digestion or fragmentation by restriction enzymes³⁴. These DNA "scissors", which are able to recognize restriction sites normally composed of enzyme-specific sequences, are considered as highly reliable tools for molecular biologists as they allow for the manipulation and design of new genetic constructs^{34,35}.

Another attractive feature of DNA is its ability to interact with other molecules, such as other nucleic acids, proteins, polymers, drugs, ions and metal compounds^{36–38}. This has made DNA a key subject in various biomedical applications, particularly in gene therapy^{39,40} and in anticancer drug treatment^{41–43}. Combined, all the intrinsic properties of DNA described above make it a very interesting material for many scientific fields.

Indeed, since the discovery of its peculiar structure, DNA has been deeply explored by scientists from the most diverse fields. The Human Genome Project^{44,45}, one of the biggest tasks ever accomplished in history, largely unveiled the mystery behind human DNA and its genes, changing forever the future of science and medicine. Parallel to this, and as a result of its physical-chemical and structural properties, scientists have also shown DNA to be incredibly versatile and excellent in the preparation of nano- to bulk materials^{46,47}. Taken as a whole, DNA has made a major mark in several interdisciplinary fields including, among others, bioinformatics^{48,49}, forensics⁵⁰, chemical engineering⁵¹, genetics^{52,53}, nuclear^{54,55}, biochemistry and biophysics⁵⁶, medicine⁵⁷, biomedical⁵⁸, nanotechnology^{59–61} and material science^{62,63}. In fact, a simple search on "DNA" in *ScienceDirect* or *PubMed* in the "Title", "Abstract" and "Keywords" fields, from "1948" to "present" provides us with a good overview of the extent in the investigation that has been carried out around DNA (Figure 3).

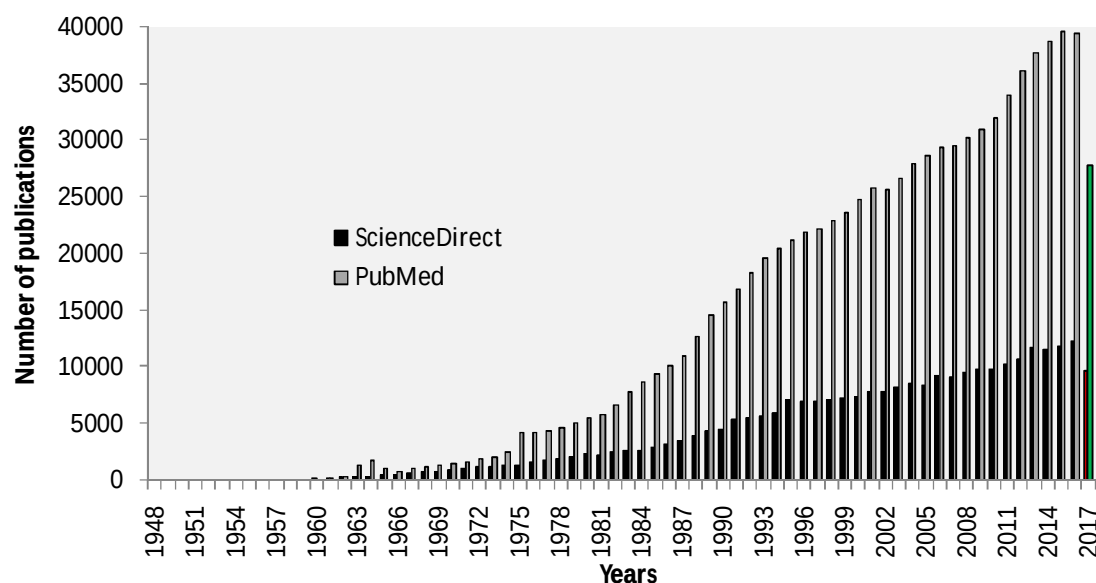


Figure 3 - DNA related published literature. Histogram showing the increasing number of publications per year in ScienceDirect.com (black) and PubMed.gov (grey) regarding the research focused on DNA.

In materials science, researchers all over the world have been using either DNA of natural origin from various sources^{64–66}, rationally designed and customized specific DNA sequences⁶⁷ or tridimensional structures⁶⁸. From nano to bulk materials, science and technology have contributed to the development of a wide range of creative DNA structures^{62,69,70}. At the nanoscale, some examples include origamis⁷¹, nanomotors⁷², dendrimers^{73,74}, polyhedra⁷⁵, tiles⁷⁶, nanocages⁷⁷, nanotubes^{78,79}, nanowires⁸⁰, nanocrystals⁸¹, nanorings⁸², nanoshells⁸³, nanomachines^{84,85}, catenanes⁸⁶, hydrogels^{87,88} and films⁸⁹. Although all very interesting, the above-mentioned DNA nanostructures are out the scope of this review. Instead, it will focus on DNA-based hydrogels and films which have also been the subject of intensive research because of their important properties and applications.

2. DNA-based hydrogels

According to the IUPAC Gold Book, a gel is a "non-fluid colloidal network or polymer network that is expanded throughout its whole volume by a fluid, [...] formed by crosslinked polymer chains or by non-linear polymerization"⁹⁰. On this basis, a gel can thus be defined as a continuous two-component dispersion made up of a structural solid phase with a liquid phase⁹¹, with a tridimensional structure capable of changing its volume in the presence or absence of the liquid phase. The solid phase can consist of polymer building blocks (monomers) or polymers chains that are physically or chemically crosslinked^{92,93}, while the liquid phase can either be water (hydrogel)⁹⁴ or an organic solvent (organogel)⁹⁵. The chemical nature of the polymer defines the gel type, where a hydrophilic

polymer constitutes a hydrogel and a hydrophobic polymer an organogel. One must bear in mind that a viscous solution that results from the dissolution of high molecular weight compounds, is not the same as a hydrogel. Viscous solutions do not have defined tridimensional structures but rather consist of entangled chains that upon the addition of water become diluted and do not undergo volume/phase transitions⁹⁶.

A hydrogel is thus made up of a tridimensional network with a mesh size in which the average size between the crosslinking points is directly related to its porosity⁹⁷. Also, hydrogels have the ability to retain large amounts of water by changing their size and/or shape thereby undergoing a volume transition. This property has been extensively addressed in many research and review papers on the topic^{98–100}. In the presence of water, hydrogels undergo expansion of the polymeric network (*i.e.* swelling)¹⁰¹, resulting in a liquid mass fraction higher than the polymeric mass fraction¹⁰². Without water, the network starts to contract (*i.e.* shrink or collapse)¹⁰¹. The degree to which this physical change occur is highly dependent on the nature and properties of the polymer chains, as well as on the crosslinking density and solution to which the hydrogel is exposed⁹⁴. Environmental stimuli, including alterations in pH, ionic strength and temperature, as well as exposure to specific molecules, may also affect hydrogel behaviour in a similar manner¹⁰³ (Figure 4, A). Hydrogel response however is not just limited to volume transitions. Depending on the chemical and physical properties and the modifications performed on the hydrogel building blocks, various other responses can be achieved including changes in shape, phase, optics, mechanics, electric fields, surface energies, recognition, reaction rates and permeation rates¹⁰⁴. When showing this behaviour, hydrogels are often referred to as stimuli-responsive or "intelligent" or "smart" materials as they are able to provide useful feedback upon receiving a specific stimulus^{100,105}.

Over the years, the body of work focused on the development of hydrogels has increased dramatically. For a comprehensive overview of the research done in the field, several review papers^{96,101–103,106–113} may be consulted. Indeed, hydrogels have been synthesized from the nano- to the macro-scale using a wide range of different materials of natural and/or of synthetic origin¹⁰⁵. Their preparation has been accomplished through chemical and physical methods, allowing for tunable properties¹¹⁴, control over the polymer molecular weight, functionalisation, crosslinking density, biocompatibility, biodegradability, mechanical strength, as well as chemical and biological response to stimuli¹⁰⁵. Due to their attractive structural and/or functional properties, hydrogels are now a widely used class of biomaterials with its most popular application, among others⁹⁴, as controlled drug delivery systems¹¹⁵ (Figure 4, B).

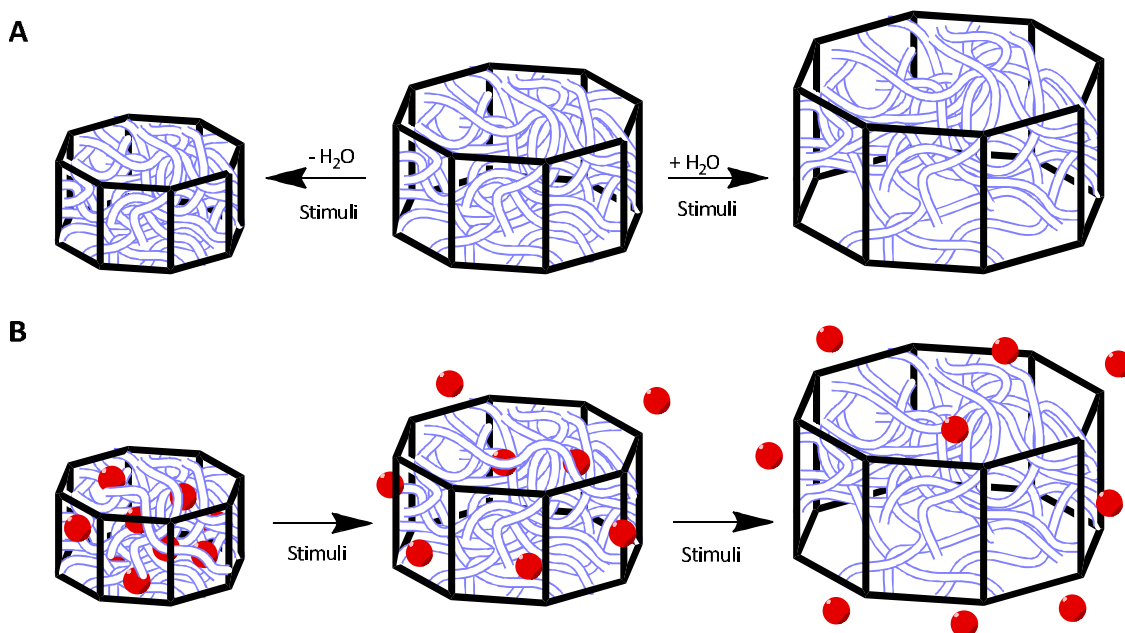


Figure 4 - Representation of hydrogels tridimensional structure and behaviour. (A) Hydrogel swelling and shrinking behaviour in the presence or absence of water and ions, that may also occur under other environmental conditions such as different temperatures, pH values or molecular species. (B) Functional/therapeutic molecule, drug or gene encapsulated *in situ* or by imbibition can be released upon swelling of the tridimensional network when promoted by specific stimuli.

In this review, one will focus on DNA-based hydrogels which include networks of different molecules chemically (Figure 5, A) or physically (Figure 5, B) crosslinked to each other, forming a tridimensional structure, where DNA acts as the main polymeric component and/or as a component responsible for distinguished hydrogel properties (usually as a crosslinking moiety). Most gels with DNA in its composition, be it at a low or at a high content, are hydrogels due to the hydrophilic nature of the molecules involved. DNA itself possess a sugar-phosphate backbone and is thus able to retain water in its structure. Importantly, as will be evident from the works highlighted along this review, both ssDNA and dsDNA molecules have been used as building blocks in DNA-based hydrogels. DNA organogels, which are much less represented in the scientific literature, usually involve the formation of tridimensional networks made up of complexes of DNA and lipids¹¹⁶. Features making DNA a good candidate for hydrogel construction include its biological function (*i.e.* genetic information storage), as well as its physical and chemical properties such as specific sequence recognition (hybridization), tunable design (sequence design), functionalisation (chemical modification for conjugation), biodegradability (enzymes) and biocompatibility (non-toxic degradation products)¹¹⁷.

DNA from different sources have been used for hydrogel preparation. From natural origin (Figure 5, C) to lab-manufactured specific sequence design (Figure 5, D), each source has its pros and cons. DNA directly extrated from organisms is less costly. Since it can be massively extracted from

tissues like from marine industry waste products¹¹⁸, it can be used in large quantities, thus being more suitable for the production of hydrogels with DNA as their main component. On the other hand, lab-manufactured DNA sequences are costly to produce. Reagents, synthesis procedures, purification and characterization are all factors that contribute to the high associated cost of producing specific DNA sequences. Despite this, synthetic DNA sequences are being used in the DNA hydrogel field either as minor (*e.g.* crosslinking bridge) or as major components as they can confer the hydrogel with specific molecular recognition and biodegradability properties¹¹⁹.

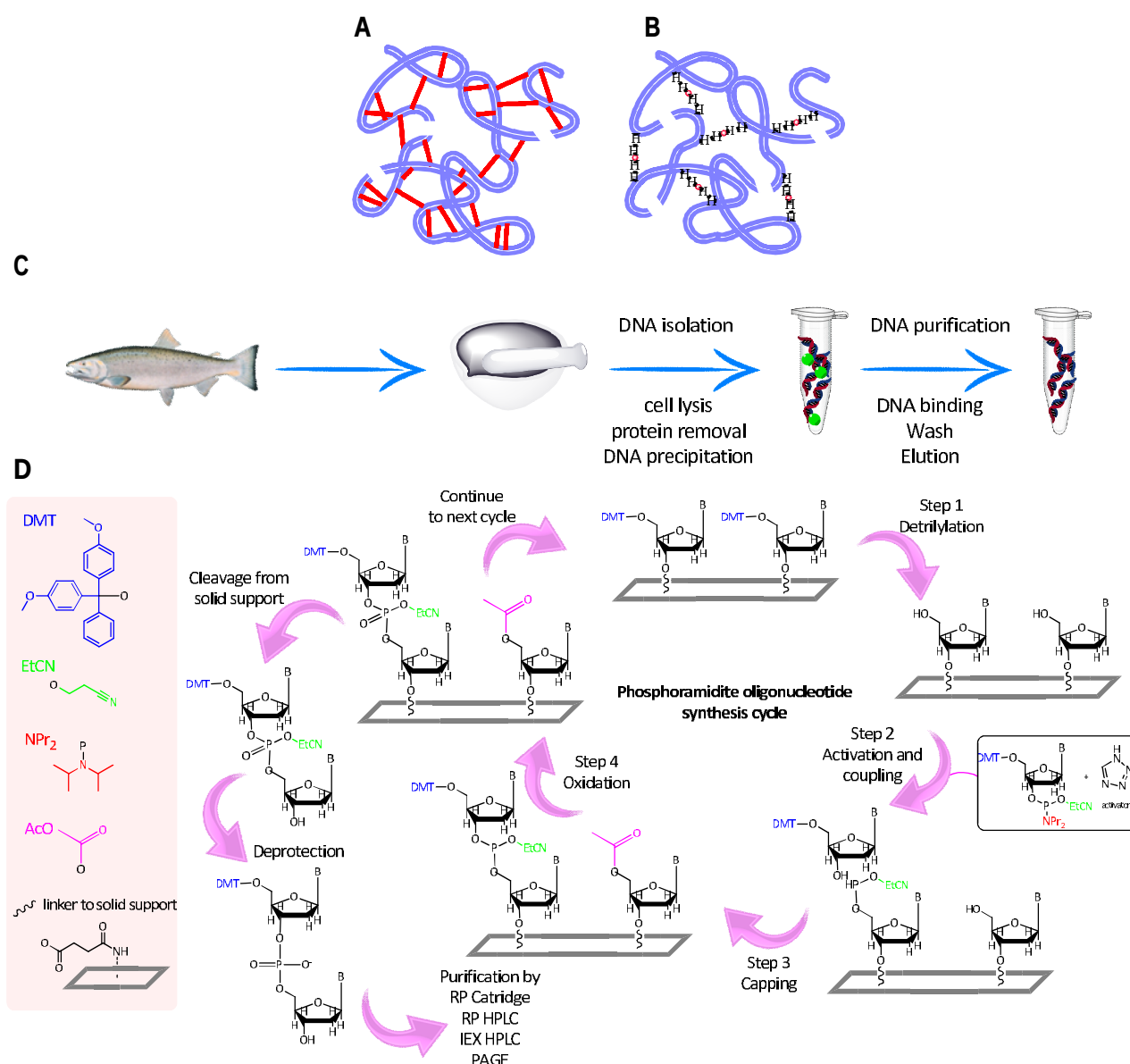
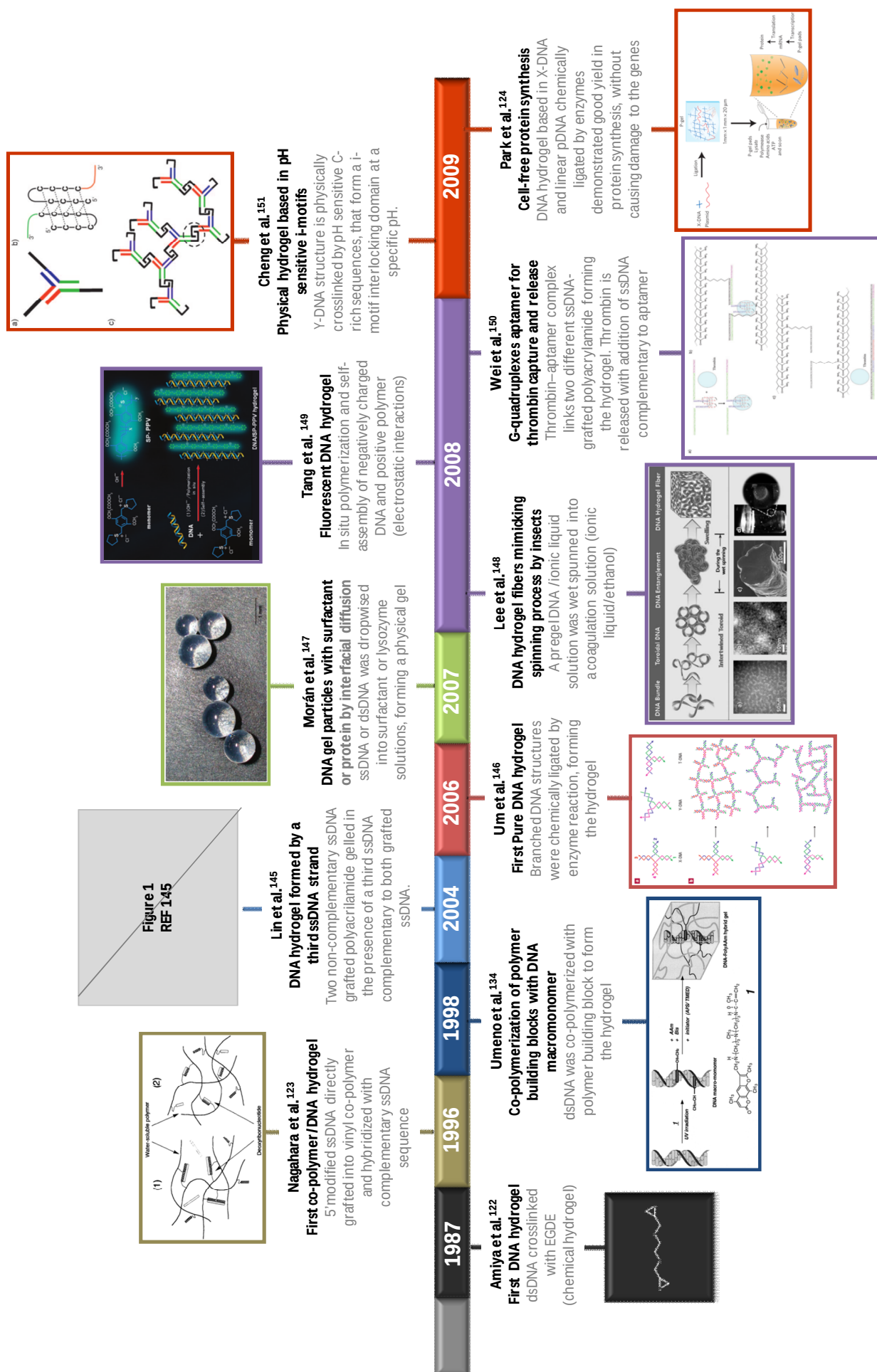
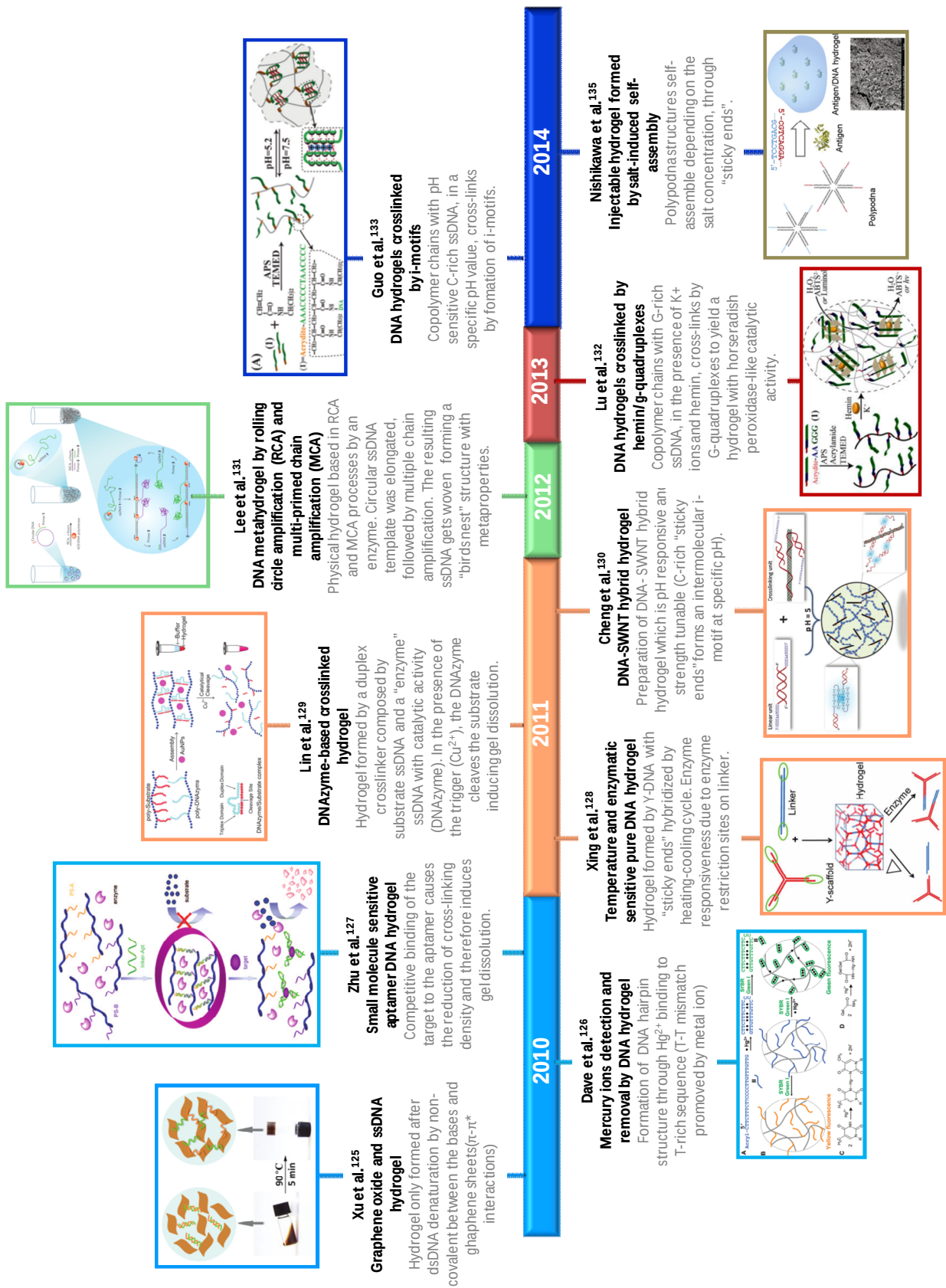


Figure 5 - Chemical and physical driven DNA hydrogels and DNA's natural and synthetic sources. (A) Chemically crosslinked DNA hydrogel with covalent bonds (red sticks) between the DNA strands. (B) Physically crosslinked DNA hydrogel with non-covalent bonds (*e.g.* hydrogen bonds) between the DNA strands. (C) Marine industry wastes are a good source to obtain DNA, after its simple isolation and purification. (D) Lab-manufactured custom DNA sequences are more laborious and costly to produce (adapter from Biomers¹²⁰).

Several types of DNA hydrogels have been prepared over the course of the years through the adoption of different assembly strategies, with the end result being hydrogels possessing distinct chemical, physical, structural and mechanical properties that may exhibit responsive behaviours towards external stimuli. Observing the timeline in Figure 6, it is possible to conclude that since 1987 the works that have been developed around this theme have increased in number and in complexity. The degree of complexity however does not concern the DNA hydrogel preparation method used. Rather, it relates to the rational design of the DNA sequences to be used for the preparation of the hydrogel (e.g. the design of DNA sequences that may form possible secondary structures, display structural and/or functional activity (i-motifs, G-quadruplexes, DNAzymes) and form a highly organized hydrogel network). These works may be broadly categorized based on the chemical nature of the hydrogel (pure or hybrid), the type of interactions established among hydrogel components (physical or chemical) or the hydrogel responsiveness behaviour upon external stimuli. Regarding the type of interactions established among hydrogel building blocks, these are of extreme importance as they strongly affect the hydrogel properties. Non-covalent bonds (physical interactions), such as *van der Waals* forces, electrostatic forces, hydrogen bonds, π - π stacking or hydrophobic interactions may give rise to hydrogels¹²¹. On the other hand, the tridimensional structure can be held together also by covalent bonds. In general, it is possible to state that hydrogels resulting from chemical bonds are more stable than the ones only based on physical interactions¹⁰¹.

In the next sections, an overview of the various DNA hydrogel categories and underlying concepts will be given. It was decided to first address the hybrid gels and only then the pure ones following the rational of starting from the simpler systems to the more sophisticated ones. In each case, representative works developed in the field over the years will be presented.





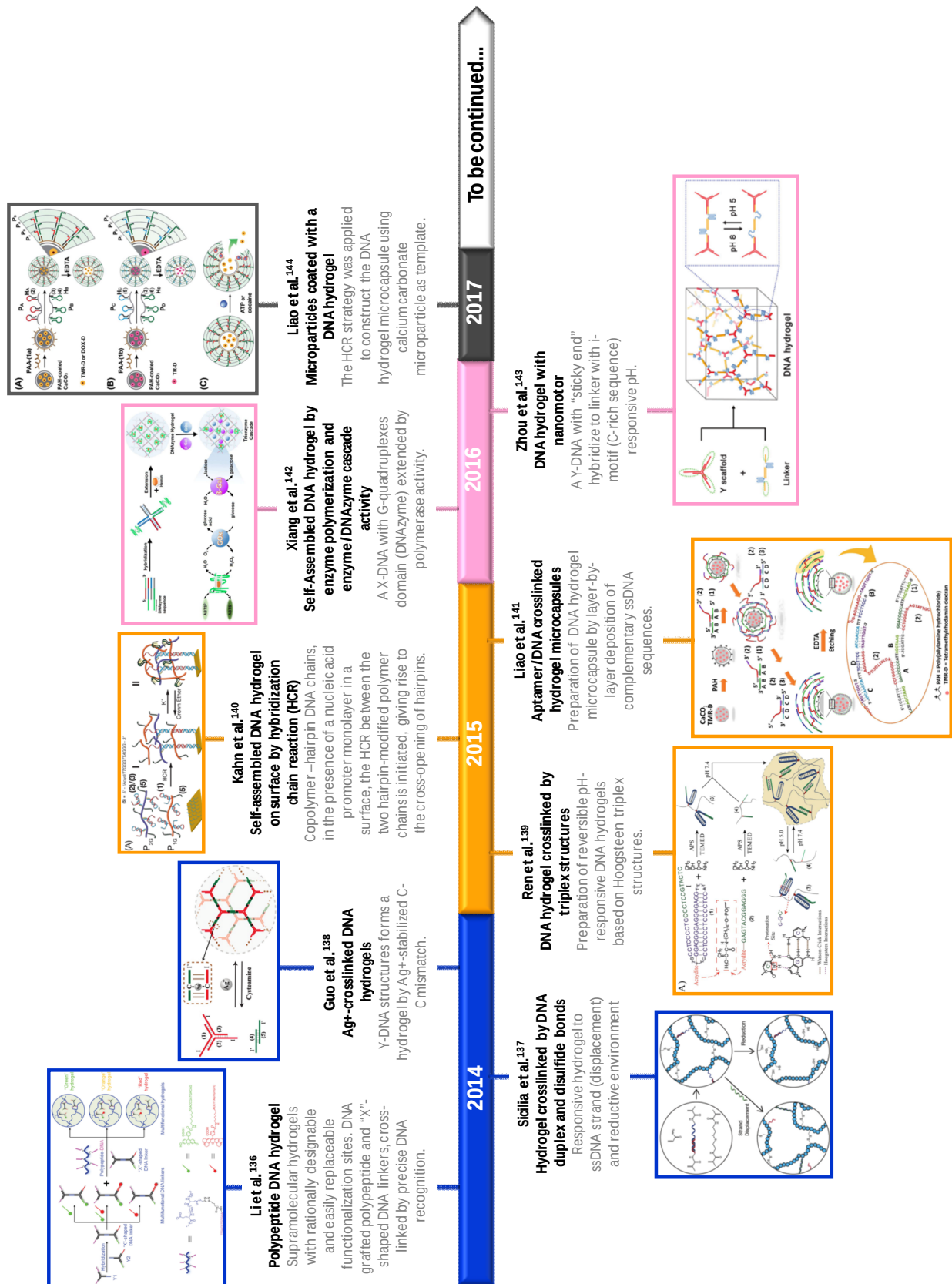


Figure 6 - Timeline of DNA hydrogels since the first reported gel in 1987 by Amiya et al. Several types of DNA hydrogels have since been produced, from pure to hybrid, using either covalent or non-covalent bonds among the hydrogel components^{122–151}. All displayed images were obtained from the corresponding references with permission of the publisher, indicated in the credit reference list.

2.1. Hybrid DNA-based hydrogels

Different approaches have been used to produce hybrid DNA-based hydrogels, combining DNA with different types of molecules and taking advantage of each of the components to create materials with interesting properties that do not exist in nature¹⁵². In these materials, DNA may appear as a main component or, otherwise, as an essential minor component that is responsible for the formation of the hydrogel (acting in the crosslinking process) and/or that endows it with special properties (e.g. chemical recognition capability and biodegradability). In the following paragraphs, several examples of these hybrid DNA-based hydrogels will be described which will be grouped according to the nature of the interactions responsible for the hydrogel formation.

Chemically crosslinked hybrid DNA-based hydrogels

Chemical crosslinking is an efficient method to produce hydrogels with strong resistance and improved mechanical properties, as well as huge and reversible volume transitions upon external stimuli¹⁵². Combined, these features are, for example, important in the biomedical field for controlled and sustained release gene/drug delivery applications. Responsible for making hydrogels of this type more stable are covalent bonds. However, in the case of some biomedical applications, labile bonds may be introduced into the hydrogel structure to improve upon its biodegradability¹⁵³.

In 1987, Amiya and Tanaka¹²² synthesized the first DNA-based hydrogel using dsDNA of natural origin from salmon tests. For this, the dsDNA molecules were chemically crosslinked with ethylene glycol diglycidyl ether (EGDE) and the volume transition of the resultant hydrogels was then studied in a water-acetone solution. Since then, this chemical crosslinking approach has been described and used by several authors^{25,88,121,122,153–174}. EGDE contains epoxide groups at both ends (it is a homobifunctional crosslinker) of its chemical structure that are able to react with nucleophiles such as the amino groups of the nucleotide bases (Figure 7). It seems that the guanine nitrogen atom at position seven (N-7) attacks the more substituted carbon of the EGDE epoxide group (nucleophilic substitution type 2 - S_N2)^{160,164}. Since EGDE has two epoxide groups, it can crosslink different points in the same DNA molecule creating intramolecular loops or it can form crosslinks between different DNA molecules leading to intermolecular polymerization.

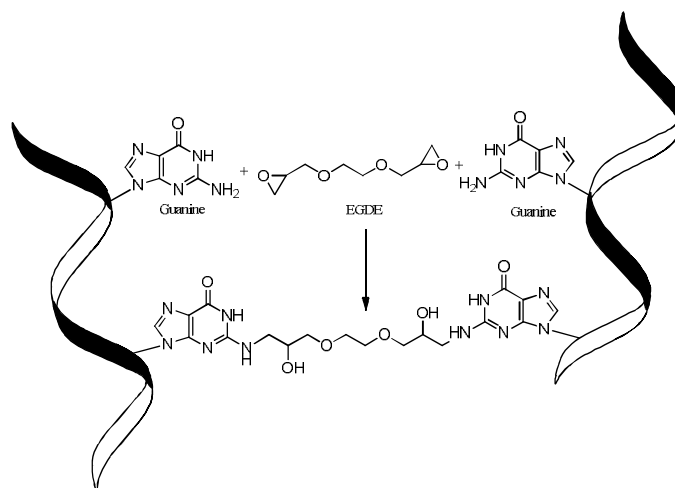


Figure 7- First chemically crosslinked DNA hydrogel. EGDE crosslinking reaction with DNA's guanine amino group, from the same or from different DNA chains.

Initially, an extremely alkaline solution of EGDE (pH $\approx$ 11) was used on its own with the DNA solution^{122,170}. Later, N,N,N',N'-Tetramethylethylenediamine (TEMED) was used in conjunction with this bifunctional crosslinker to adjust the pH to 9¹⁷¹ and to function as a catalyst to initiate the crosslinking reaction with DNA. DNA hydrogels have also been prepared using ssDNA¹⁷⁴ and circular plasmid DNA (pDNA) by this method¹⁶⁰. In earlier works, it was mentioned that this procedure did not denature the DNA double helix to the point of losing the double stranded conformation to a single stranded one^{154,172}. Based on the reaction mechanism (*i.e.* epoxide group with guanine amine) and later statements²⁵, some degree of denaturation must occur at the crosslinking points as a result of the disruption of the C-G hydrogen bonds. This is attributable to the antagonist EGDE:TEMED pair, where in the presence of TEMED, denaturation (breakage of helix hydrogen bonds) and inter and intra crosslinking (gelation) is by EGDE. The question here is to what extent denaturation occurs such that it leads to complete loss of conformation. It was concluded by Topuz *et al.*²⁵ that dsDNA denaturation under these conditions depends on the concentration of DNA used. At higher DNA concentrations, a higher concentration of counterions will also be present, which will stabilize the dsDNA conformation. In contrast, at lower DNA concentrations, denaturation will be favored. Possibly, since in the earlier works higher DNA concentrations were used, loss of DNA conformation was not observed.

Bulk DNA hydrogels obtained using this method have been extensively studied in terms of their stimuli-responsive behaviour in the presence of different composition aqueous solutions^{122,154,171}, their electrical response in the presence of DNA binding substrates¹⁷⁰, and their mechanical properties (such as stiffness and elasticity) upon thermal treatment^{25,169} and strain¹⁵⁷. All these characterizations have contributed to our understanding of the intrinsic and dynamic

properties of DNA hydrogels, particularly in the context of biomedical applications (later addressed) and environmental issues such as carcinogenic pollutants^{159,162,163}.

An interesting direction was pointed out by Zinchenko's group^{164,166,167} that takes advantage of DNA's affinity for noble and transition metals. In their work, the same EGDE crosslinked DNA hydrogel was initially prepared and then used as a "reactor" for the synthesis of catalytically active ultrasmall gold nanoparticles (AuNPs). The procedure consisted of immersing the hydrogel in a solution of HAuCl_4 until equilibrium state was achieved, followed by the *in situ* reduction of Au^{3+} by NaBH_4 (Figure 8, A-B). The AuNP size could be controlled by changing the pH of the reduction solution (Figure 8, C). Such metalized hydrogels have been utilized in catalytic applications for environmental waste removal (Figure 8, D).

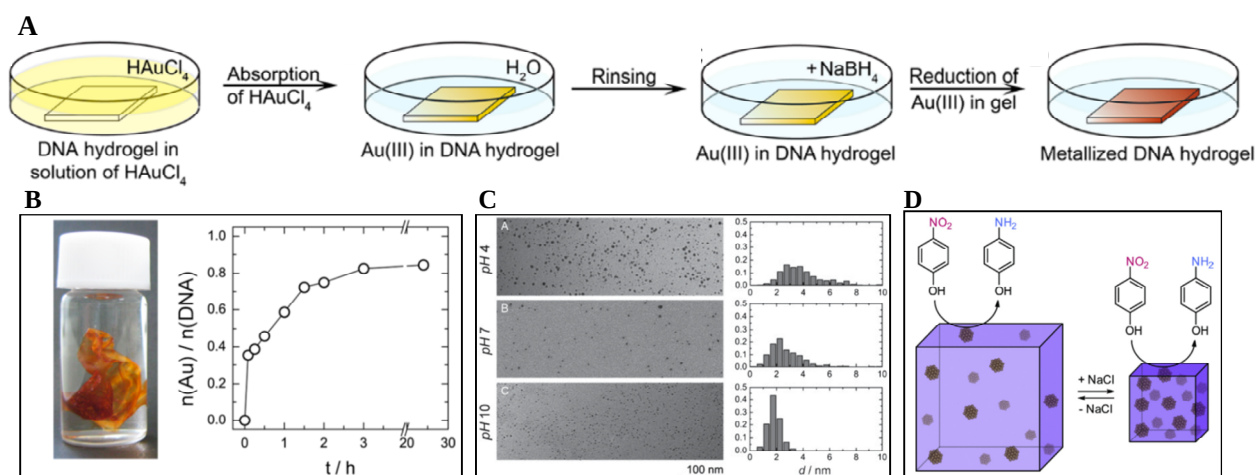


Figure 8 - DNA hydrogel AuNP catalytic reactor for environmental waste removal. (A) Incubation of DNA hydrogel in HAuCl_4 solution; absorption of Au(III) ions into the hydrogel; reduction of the gold ions by the NaBH_4 reduction agent; AuNP metallized DNA hydrogel¹⁶⁶. (B) Metallized DNA hydrogel in solution and the time vs. HAuCl_4 uptake by the DNA hydrogel¹⁶⁶. (C) TEM images and size distribution of the AuNPs in hydrogels at different pHs¹⁶⁴. (D) p-nitrophenol reduction to aminophenol catalyzed by the AuNPs in the DNA hydrogel, with simultaneous volume transitions in different NaCl concentrations¹⁶⁷. Adapted/reprinted with permission from ref. 164, Copyright © 2014 John Wiley & Sons, Inc., ref. 166, Copyright © 2014 American Chemical Society and ref. 167, Copyright © 2015, Elsevier.

Costa and coworkers have been working on EGDE crosslinked DNA hydrogels for more than a decade and have contributed greatly to the field^{88,121,153,155,156,158,160,161,165,168,172–174}. Their studies have focused on the extensive analysis of the properties and behaviour of DNA hydrogels under various conditions. Initially, their work involved characterizing bulk hydrogels made up of EGDE crosslinked with dsDNA in terms of DNA conformation and swelling behaviour in the presence of several solutes (e.g. salts, surfactants, polyamines). The swelling behaviour of hydrogels prepared using either dsDNA, ssDNA (for evaluation of strand flexibility) or pDNA at different crosslinking degrees (mesh sizes) was also addressed in their research, allowing for the creation of perfect tunable environments for the encapsulation of small or big molecules that can be released in a controlled manner. They

gradually transitioned from bulk to micro- and nanoscale hydrogels using pDNA, thus paving the way for the use of such materials in drug and gene delivery applications.

DNA hydrogels are usually prepared in electrolyte-containing solutions. As a matter of fact, most of the dsDNA of natural origin used to prepare DNA hydrogels has sodium ions as counterions. The presence of these counterions in DNA are known to play a role in stabilizing the double helix structure and preventing DNA denaturation¹⁷¹. Thus, when prepared, the DNA hydrogel network is surrounded by ions that allow it to be maintained in an equilibrium state with a stable volume. However, hydrogels are known to undergo reversible volume transition. This occurs when the gel comes into contact with a liquid phase having a different composition to the one initially used to prepare it. The main cause for this volume transition is due to osmotic pressure (*i.e.* the difference between internal and external ion concentrations)¹⁷⁵. Thus, if the solution outside the hydrogel presents a higher ion concentration, the hydrogel will shrink, otherwise, it will swell. Importantly, the swelling/deswelling behaviour of DNA hydrogels has been shown to depend on the swelling pressure, that is, on the chemical and structural properties of the hydrogel network.

Several types of co-solutes were used in the studies conducted by Costa *et al.*^{121,172,173}. When using inorganic salts with different cation valency (Figure 9, A-C), the swelling/deswelling process was driven by osmotic pressure and the shrinking degree was found to be lower with smaller ionic radius and valency. In the case of polyamines such as spermine (SP) and spermidine, as well as cationic macromolecules such as chitosan (CHI), lysozyme, poly-(L-lysine) (PLL) and poly-(L-arginine) (PARG) (Figure 9, D), the swelling/deswelling of the DNA hydrogels was driven by electrostatic interactions and the shrinking degree was higher with highly charged molecules. When using surfactants (Figure 9, E), the swelling/deswelling behaviour of the hydrogels was driven by hydrophobic interactions and diffusion, with the shrinking degree being higher with positively charged and smaller hydrophobic tail surfactants. The effect of DNA conformation and size on the swelling behaviour of the hydrogel was also investigated (Figure 9, F)¹⁷⁴, with the degree of swelling being higher for ssDNA relative to dsDNA. This was attributed to differences in charge density, chain flexibility and hydrophobicity.

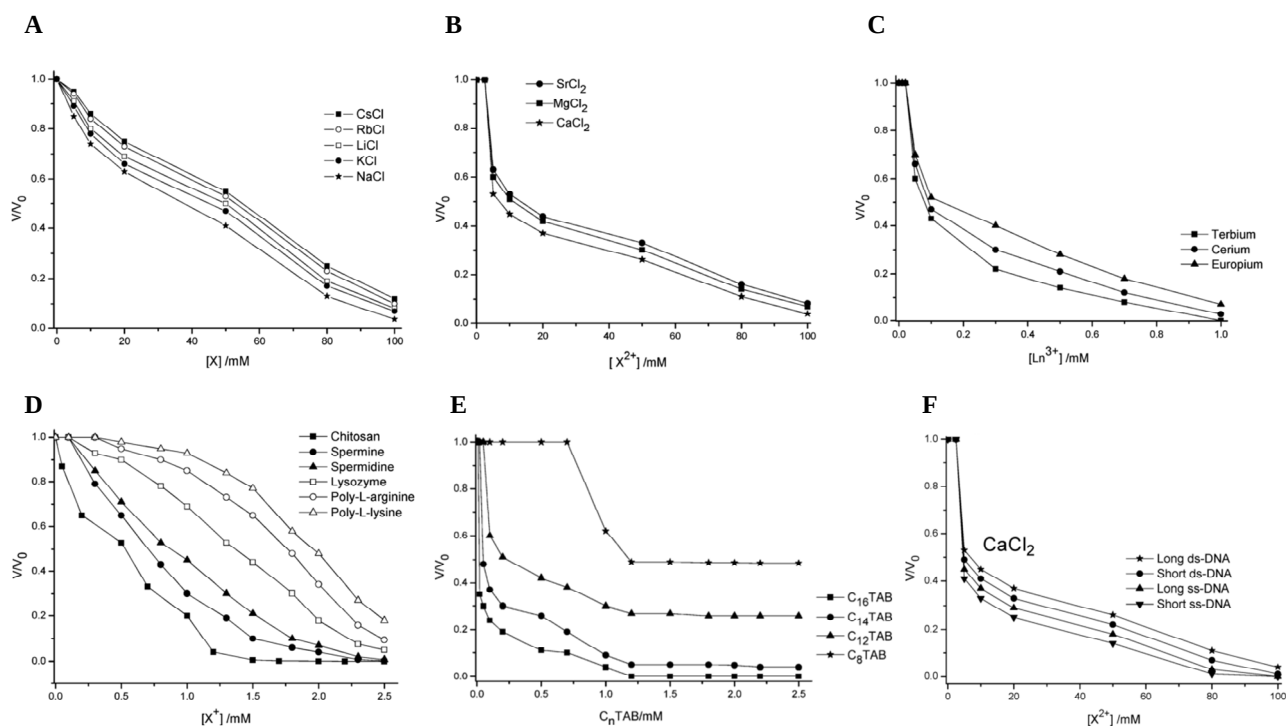


Figure 9 - Swelling behaviour of EGDE crosslinked DNA hydrogels in the presence of different solutes. Swelling isotherms (V/V_0) for dsDNA hydrogels (1% EGDE) immersed in different solutions of (A) monovalent (0-100 mM), (B) divalent (0-100 mM), and (C) trivalent ions (0-1.0 mM), all at 25°C and pH 9¹⁷³. (D) dsDNA hydrogels (1% EGDE) immersed in different polyamine solutions (0-2.5 mM, at 25°C and pH 6.5)¹⁷³. (E) dsDNA hydrogels (1% EGDE) immersed in different length surfactants (0-2.5 mM, at 25°C and pH 9)¹⁷³. (F) From the different co-solutes tested, CaCl_2 was chosen to demonstrate the swelling behaviour of the hydrogels produced when using several different DNA types (long vs. short and ssDNA vs. dsDNA)¹⁷⁴. Adapted/reprinted with permission from ref. 173 and 174, Copyright © 2007 American Chemical Society.

Closer analysis of the swelling behaviour of ssDNA hydrogels shows that the process is driven by hydrophobic interactions resulting from the hydrophobic nature of the nitrogen bases and by electrostatic interactions attributable to the negatively charged DNA phosphate backbone. This behaviour thus depends on the hydrophobic interactions and diffusion of the surfactant in the gel, with the diffusion of longer hydrophilic tail surfactants being more difficult inside the gel due to hydrophobic interactions¹⁵⁵. In terms of DNA strand flexibility, it is known that as a linear polymer, DNA has some degree of flexibility that is length, conformation and ion-dependent. This is related to DNA's persistence length (*i.e* the minimum length at which a polymer strand is considered flexible)¹⁷⁶. The higher the value here, the less flexible (or more rigid) the polymer chain is. In the case of dsDNA, the persistence length is ~ 50 nm (150 bp), while for ssDNA the value is 1 nm¹¹⁹. Thus, DNA strands shorter than their persistence length are considered to be rigid rods. On this basis, ssDNA hydrogels are more flexible and "swellable" than dsDNA hydrogels. Also, the degree of crosslinking in a hydrogel will influence its "swellability". This is mainly attributable to contour length (L), a parameter related to the distance between crosslinking points in a network^{23,25}. As the degree of crosslinking increases, the hardness of the hydrogel will increase and the swellability decrease as

there is less freedom to swell (*i.e.* swelling is restricted by the contour length).

The utilization of EGDE as a crosslinker has also opened the door for the controlled release of molecules as it is photodegradable by UV radiation^{153,156}. DNA hydrogels crosslinked with different EGDE concentrations have been shown to undergo weight loss after light exposure, mainly due to the release of DNA. Thus, the loss of the chemical bonds responsible for hydrogel integrity leads to a decrease in gel stiffness and an increase in swelling. Costa *et al.*¹⁵⁸ later evaluated the light triggered release of solutes such as bovine serum albumin (BSA), lysozyme and fluorescein isothiocyanate-dextran (FITC-dextran) from different DNA hydrogels (Figure 10). Overall, a controlled release of the solutes from the hydrogels was observed to be dependent on the crosslinking density, swelling degree and hydrodynamic size of the molecules.

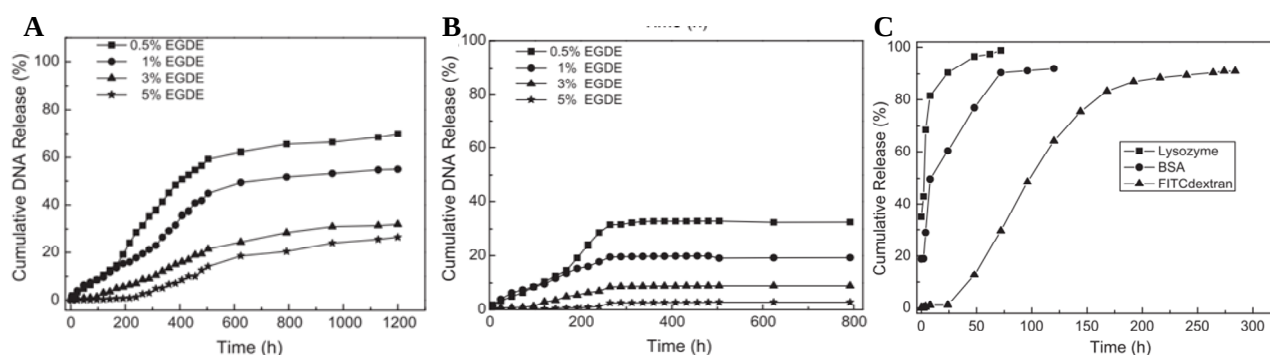


Figure 10 - DNA and bigger molecules release from EGDE crosslinked DNA hydrogels. Cumulative release of DNA from different DNA hydrogels (0.5, 1, 3 and 5% EGDE) as a function of time, after (A) light exposure and (B) in the absence of light. (C) Cumulative release of lysozyme, BSA and FITC-dextran from 0.5% EGDE crosslinked DNA hydrogels, as the hydrogel mesh increases with time (evolution of degradation)¹⁵⁸ Adapted/reprinted with permission from ref. 158, Copyright © 2011 Elsevier.

Combining the advantages of hydrogels (*i.e.* tridimensional structure, high water content, biocompatibility, hydrophilic drug loading capacity and tunable properties) with those of DNA (*i.e.* genetic information carrier, hydrophobic and intercalating drug loading ability), Costa *et al.* also developed materials for dual function applications using pDNA^{160,161,165}. Their contribution in this domain is well summarized in a review¹⁷⁷. Briefly, pDNA microgels were obtained by chemically crosslinking pDNA encoding the p53 gene with different concentrations of EGDE. Because of the EGDE crosslinker, the resulting hydrogels were photodegradable¹⁵³ making them suitable for drug and gene co-delivery. It is evident that the DNA in this study has a dual function: structural (as the main component of the gel) and functional (after gel degradation the pDNA will be internalized and subsequently expressed by the cells). The work developed came to the following conclusions (Figure 11): (i) porous and transparent microgels were obtained, (ii) the pDNA double helix was preserved after the crosslinking reaction, (iii) the microgels were biocompatible due to the presence of the DNA, with an increase in crosslinking density leading to an increase in toxicity, (iv) microgel

swelling behaviour depended on the polymer network parameters, including crosslinking density, hydrophobic/hydrophilic balance, charged groups, flexibility and interaction with solutes, (v) a higher percentage of crosslinker resulted in more resistance to swelling (0.5% EGDE crosslinked hydrogels had less degrees of freedom relative to 0.2% EGDE crosslinked gels), (vi) in the presence of monovalent electrolytes, microgels contracted due to the shielding effect and to osmotic pressure, (vii) microgel contraction was more pronounced in the presence of a smaller ionic radius (e.g. $\text{Na}^+ > \text{K}^+ > \text{Rb}^+ > \text{Cs}^+$), divalent salts ($\text{CaCl}_2 > \text{MgCl}_2 > \text{SrCl}_2$), high charge molecules ($\text{CHI} > \text{SP} > \text{spermidine} > \text{lysozyme}$) and cationic surfactants (at higher concentrations, the surfactants substituted the microgel counterions and self-assembled with the DNA network, leading to aggregation and finally to gel collapse), (viii) photodegradability upon exposure to light broke the hydrogel crosslinks and increased the network mesh size, making drug and gene release simply easier (0.2% EGDE content showed a slower release than 0.1% EGDE), (ix) pDNA microgels could be prepared by inverse microemulsion polymerization with EGDE as crosslinker (smaller size microgels were obtained using a lower EGDE content), (x) small microgels demonstrated a good DNA and doxorubicin (DOX) release that could be enhanced by photodisruption, (xi) cell viability reduction had a stronger effect when the drug and the p53 tumour suppressor gene were co-delivered. The work performed in this study stands out, particularly since very few DNA hydrogel based studies evaluate dual drug and gene delivery.

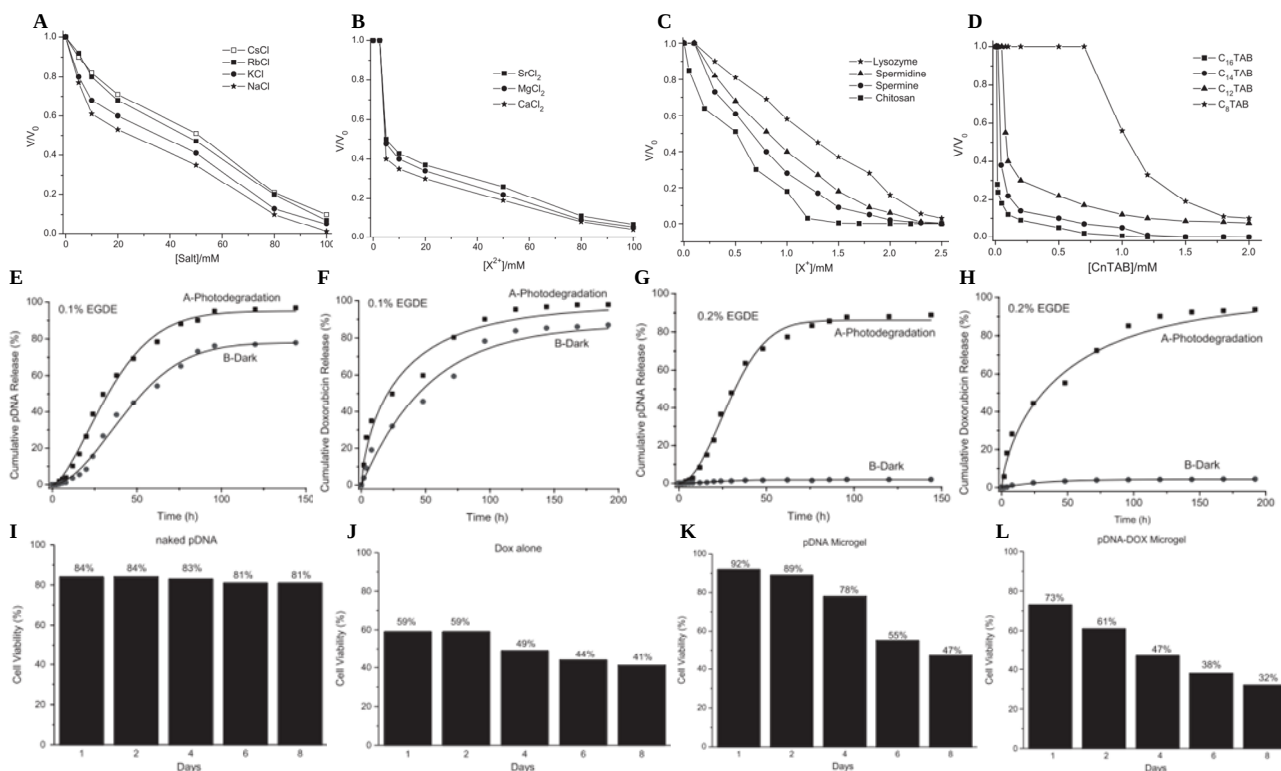


Figure 11 - Swelling behaviour of EGDE crosslinked pDNA microgels in the presence of different solutes, conditions, as well as cell viability.. Swelling isotherm (V/V_0) for pDNA microgels (0.2% EGDE) immersed in different solutions of (A) monovalent ions (0-100 mM), (B) divalent ions (0-100 mM), (C) polyamines (0-2.5 mM) and (D) surfactants (0-2 mM), all at 25°C and at a pH of 8.5 for (A) to (C) and a pH of 6.5 for (D)¹⁶⁰. Cumulative release of (E) pDNA from a 0.1% EGDE microgel, (F) DOX from a 0.1% EGDE microgel, (G) pDNA from a 0.2% EGDE microgel and (H) DOX from a 0.2% EGDE microgel, all as a function of time, in the dark and after irradiation with a 400 nm light¹⁶¹. Viability of HeLa cells after transfection with (I) naked pDNA, (J) DOX alone, (K) a 0.1% (w/v) EGDE pDNA-lipofectamine microgel and (L) a 0.1% (w/v) EGDE pDNA/DOX-lipofectamine microgel, with cell viability being determined by MTT assay after 1, 2, 4, 6 and 8 days exposure¹⁶⁵. Adapted/reprinted with permission from ref. 160, Copyright © 2011 Elsevier, ref. 161, Copyright © 2012 John Wiley & Sons, Inc. and ref. 165. Copyright © 2013 Elsevier.

Costa *et al.* recently adopted an alternative approach to produce pDNA nanogels^{88,168}. The stimulative-responsive swelling and release behaviour properties were also analyzed for each of the nanogels prepared here (Figure 12). To produce the nanogels, pDNA was combined with three different polyamines, namely SP, protamine (PrS) and poly(ethyleneimine) (PEI), to promote its condensation *via* electrostatic interactions. In each case, this process was performed prior to (protocol I) or during (protocol II) crosslinking with EGDE at different concentrations. pDNA condensation prior to crosslinking resulted in smaller, positively charged nanogels, all favorable parameters for cell uptake¹⁷⁸. Volume transition studies were also carried out by placing the nanogels either in water or in sodium chloride to monitor their swelling and shrinking behaviours, respectively. It was found that nanogels with higher crosslinking density were more resistant to the osmotic swelling force. Nevertheless, their swelling behaviour could be modulated by ions. The controlled

and sustained release of genes and anticancer drugs such as DOX, epirubicin (EPIR) and paclitaxel (PTX) only occurred after UV radiation, without initial burst. The cell viability data corroborated these results. Overall, this study demonstrated the co-delivery of the p53 tumour suppressor gene and anticancer drugs with synergistic effects.

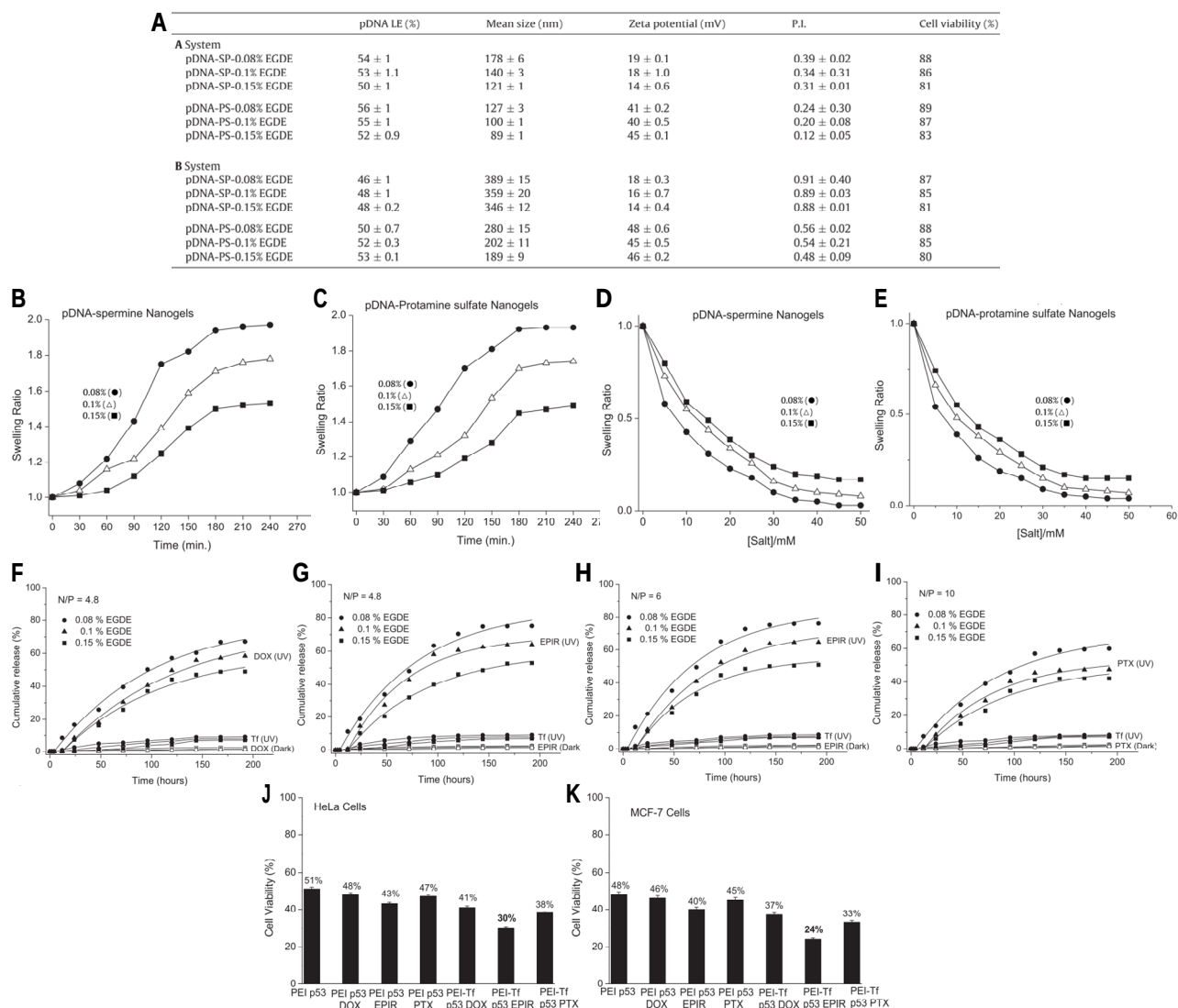


Figure 12 - Nanogels properties, stimuli-responsive swelling/release behaviour and cytotoxicity. (A) Table summarizing the properties of SP and PS pVAX1-LacZ nanogels with condensation prior to (protocol I-A) or during (protocol II-B) crosslinking. pDNA loading efficiency (LE), hydrodynamic mean size (nm), zeta potential (ZP) and polydispersity index (PI) are indicated⁸⁸. Swelling ratio of different pDNA nanogels, namely, (B) pDNA-SP in water, (C) pDNA-PS in water, (D) pDNA-SP in NaCl and (E) pDNA-PS in NaCl, in each case prepared using 0.08, 0.1 and 0.15% EGDE⁸⁸. Cumulative release of (F) DOX, (G and H) EPIR and (I) PTX from 0.08, 0.1 and 0.15% EGDE crosslinked PEI-transferrin (Tf) p53 nanogels, in the dark or after irradiation with UV light (400 nm), as a function of time. In each case, different N/P ratios were tested when preparing the gels¹⁶⁸. Viability of Hela (J) and MCF-7 (K) cells 8 days post-exposure with 0.08% EGDE crosslinked PEI p53, PEI p53 DOX, PEI p53 EPIR, PEI p53 PTX, PEI-Tf p53 DOX, PEI-Tf p53 EPIR and PEI-Tf p53 PTX nanogels¹⁶⁸. Adapted/reprinted with permission from ref. 88, Copyright © 2014 Elsevier and ref. 168, Copyright © 2015. Elsevier.

An interesting study has reported the use of glutaraldehyde (GA) as a crosslinker to obtain hydrogels containing DNA¹⁷⁹. GA is well known for its crosslinking ability, with its major application being its use as a reagent for biological sample fixation and staining procedures¹⁸⁰. Indeed, it is a bifunctional chemical crosslinker with two aldehyde groups that react with the primary amino groups of proteins (Figure 13), covalently bonding different molecules¹⁸¹. In this work, DNA and gelatin were mixed and crosslinked with different concentrations of GA giving rise to hydrogels which were responsive to pH¹⁷⁹. The authors concluded that GA crosslinked gelatin and that a semi-interpenetrating network (semi-IPN) was actually obtained. Higher DNA contents were responsible for an increase in hydrogel permeation as concluded by experiments using cimetidine as a model molecule to evaluate the material permeation behaviour.

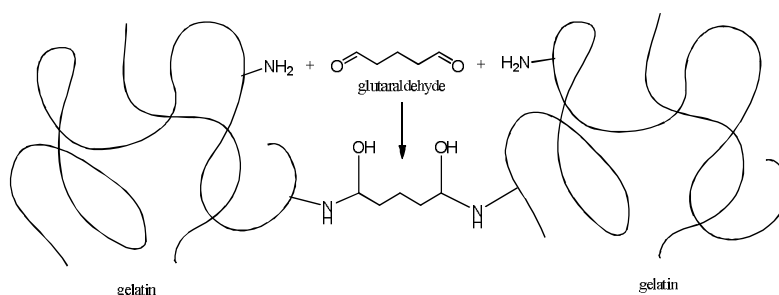


Figure 13 - GA crosslinking reaction with primary amino groups. Gelatin or other amine-containing polymers can undergo this reaction, which can occur within the same or between different polymer chains.

Hybrid DNA-based hydrogels crosslinked by specific base-pair recognition

To date, polymer grafting with ssDNA molecules (or dsDNA ending in strands with unpaired nucleotides) has been the most used approach to produce hybrid DNA-based hydrogels. Here, ssDNA is covalently linked along the length of a flexible polymer chain (resulting in a structure with a comb-like topology) and hydrogel formation is achieved by hybridization of complementary ssDNA strands, that is, by base pair recognition. Polymer grafting can be achieved either by directly grafting 5'-modified DNA molecules onto a prefabricated polymer chain or by copolymerizing the modified DNA strands with the polymer building blocks¹⁵². Each approach has its advantages. Direct grafting, for example, allows for polymer molecular weight controllability, while copolymerization avoids extra preparation and purification steps. Most of the papers published on hybrid DNA-based hydrogels crosslinked by specific base pair recognition have poly(acrylamide) (PA) as the polymeric backbone^{123,134,137,182–200}. A few other different polymer chains were used for hybrid DNA-based hydrogel preparation, including water-soluble poly(N,N-dimethylacrylamide-co-N-acryloyloxysuccinimide) (pDMA-co-NAS)¹²³, biocompatible poly(ethylene glycol) diacrylate (PEG-

diacrylate)²⁰¹, thermosensitive poly(N-isopropylacrylamide) (pNIPAM)^{133,202,203}, polypeptide poly(L-glutamic acid-co- γ -propargyl-L-glutamate) (p(LGA-co-PLG))^{136,204} and PEGylated human serum albumin (HSA) (PEG-HSA)²⁰⁵.

Two different strategies can be used for the preparation of hybrid DNA-based hydrogels relying on ssDNA-grafted polymers and complementary sequence hybridization (Figure 14, A): (i) hydrogel formation by the direct hybridization of separate polymer chains that already have complementary ssDNA strands grafted onto them or (ii) hydrogel formation using a third ssDNA "linker" that is complementary to two ssDNA-grafted polymer chains. The mechanism involved in the formation of the gels is thus physical, involving hydrogen bonds between the DNA strands and the establishment of stable duplexes. The resulting hydrogels are thermoreversible^{123,145}, meaning that an increase in temperature above the T_m of the DNA will result in denaturation of the crosslinked dsDNA forming the gel, which in turn will free the DNA-grafted polymers into solution (Figure 14, B(i)). Dictating this so-called gel-sol transition is the temperature at which the DNA strands denature, which in itself can be controlled by the length and the sequence composition of the DNA strands^{19,152}. Another common way to dissolve the hydrogels is through the use of a DNA displacement reaction (DDR) (Figure 14, B(ii))²⁰⁶. To achieve this, a toehold must be included in the crosslinker DNA strand. This toehold, which corresponds to a small sequence that will remain as a single strand after hydrogel formation, can hybridize with a displacement DNA strand^{145,183}. In the case of such an event, the displacement DNA strand will compete with and then force the detachment of the DNA linker, and consequently turn the hydrogel into a solution of free DNA-grafter polymers.

The pioneers in DNA-grafted polymer hybridization induced-gelation were Nagahara and Matsuda¹²³ in 1996. Succinimide-copolymers were first grafted with 5'-amino modified ssDNA. Gelation was then achieved either upon hybridization between two complementary ssDNA-grafted polymers or by hybridization of a ssDNA-grafted polymer with complementary ssDNA. In both cases, the gels were shown to be thermoreversible, swelled in the presence of water and hybridized spontaneously without increasing the temperature. The fact that temperature increase was unnecessary for promoting hybridization has opened the doors for molecule encapsulation without side reactions or inactivation and for the *in situ* injection of hydrogel precursors⁴⁶. This work clearly demonstrated the two different strategies adopted to obtain DNA hydrogels discussed above. In fact, to date, many other research groups have used this strategy with different chemical functionalisations and methods^{123,126,127,129,133,134,136–140,144,145,150,182–195,197–202,204,205,207–222}.

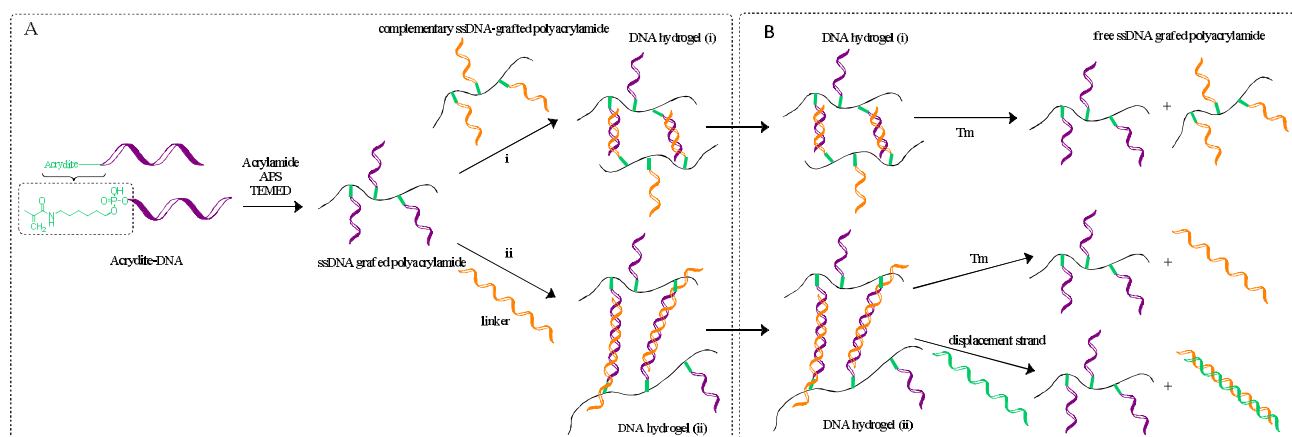


Figure 14 - DNA hydrogels prepared with ss-DNA grafted polymers. (A) Synthesis of DNA hydrogels, where ssDNA-grafted polyacrylamide is first prepared through the copolymerization of ssDNA with an acrylamide gel precursor using TEMED (catalyst) and APS (initiator). DNA hydrogel formation then occurs (i) when the ssDNA-grafted polyacrylamide is in the presence of a complementary ssDNA-grafted polymer or (ii) when a complementary ssDNA strand linker combines the ssDNA-grafted polyacrylamide with the same or different ssDNA-grafted polymers. (B) Both DNA hydrogel types shown in (A) are thermoreversible. In the case of the DNA hydrogel in (ii), if a "toehold" is included in the ssDNA strand linker, a fourth displacement ssDNA strand may compete with it eventually leading to gel disassembly.

Several other studies have focused on the preparation of hydrogels by direct hybridization, always keeping in mind their final application for DNA ligand binding and controlled drug delivery. All the possible variations and functionalities that may be used to improve upon the properties and behaviour of the formed hydrogels were taken into account in these works^{132,133,137–140,144,183–185,189,191,192,203,207,220}.

Moreover, the interaction and stability properties of hybridized ssDNA strands, controlled by the number of hybridized nucleotides, have been extensively investigated in order to determine their influence on pore size^{137,183–185} and thus their impact on hydrogel swelling behaviour. Gawell and Stokke¹⁸⁴, for example, published an interesting work showing the possibility of using DNA hybridization to modulate porosity in hydrogels made up of acrylamide and bisacrylamide. Here, the polymeric network was already reticulated but the hybridization of ssDNA strands grafted in the material allowed the control of the pore size. They used three different strategies to control the swelling behaviour of a hydrogel network that was formed by copolymerization of a polymer with two ssDNA strands, A1 and A2. As illustrated in Figure 15, when the complementary base pair sequences in A1 and A2 hybridize, a DNA bridge forms and limits the pore size of the hydrogel. Accordingly, some rigidity and a limited swelling behaviour is displayed in these hydrogels. The introduction of oligonucleotide probes, P1 and P2, which have complementary base pair sequences to A1 and A2, respectively, will allow for the displacement of the hybridized A1/A2 segments. This in turn will result in an increased flexibility and swellability in the hydrogel, which is limited to a lesser extent by the partial DNA bridge. Lastly, when the hybridized A1/A2 segments are completely

interrupted and only A1/P1 and A2/P2 bridges form, the hydrogel pores become more flexible and swellable. At this point, the gel's behaviour is only limited by the polymer network itself. In fact, the authors conclude that this material could be suitable for applications like smart drug release systems, sensors and logic valves in microfluidics and actuators.

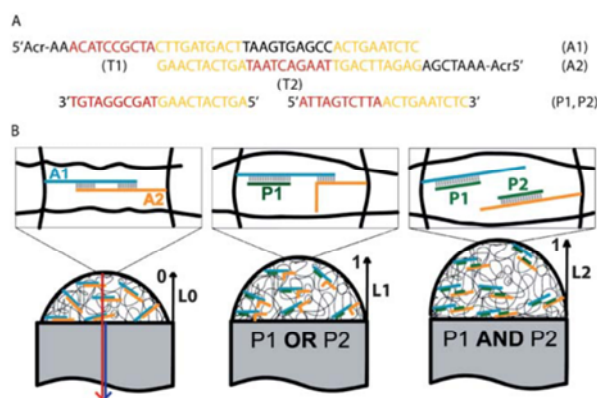


Figure 15 - DNA hydrogel with tunable swelling behaviour. (A) Hydrogel preparation where sequences of ssDNA strands (A1, A2) are integrated as crosslinkers into the network and complementary oligonucleotide probes (P1, P2) are used for inducing swelling readjustment. The A1–A2 pair is designed with sequence complementarity in two regions (yellow). (B) Interactions between ssDNA crosslinks in A1–A2 hydrogel and incorporation of P1 and/or P2 complementary probes that induce readjustment of the hydrogel swelling¹⁸⁴. Adapted/reprinted with permission from ref. 184, Copyright © 2011 The Royal Society of Chemistry.

Furthermore, the richness of DNA structures beyond duplexes has been deeply explored to modulate the behaviour of DNA-based hydrogels, which is well exemplified by the work of Willner's group that has been investigating DNA hydrogels since 2013. To date, their work has focused on taking advantage of the swelling properties of polymer hydrogels and making use of DNA's ability to crosslink either by the formation of duplexes (e.g. A-T and C-G Watson-Crick interactions; base pair mismatch), triplexes (e.g. T-A**T*, T-A**A*, C-G**G* and C-G**C*⁺Hoogsteen interactions) or specific DNA structures (e.g. i-motifs which are pH-dependent; G-quadruplexes which are ion-dependent) *via* ion stabilization^{132,133,138–140,144,189,191,192,203,207,220}. Not only do the various DNA-based formations mentioned above play an important role in the construction of the hydrogel structure, but they also contribute to the stimuli-responsive behaviour of hydrogels under specific environmental conditions.

As part of their research, Willner and coworkers produced hybrid DNA hydrogels by crosslinking partially complementary strands grafted on polymer chains or pure DNA structures. Due to the presence of a C-C mismatch on the complementary strands (Figure 16, A-B), the DNA duplexes were found to be unstable. As a result of DNA's propensity for metal ions, Ag⁺ ions were added to the duplexes leading to the formation of stabilized DNA-metal complexes through a C-Ag⁺-C bridge^{138,223}. Comparison of the Ag⁺-stabilized DNA hydrogels revealed that both the pure and the hybrid gels had

similar stiffness¹³⁸. However, the hybrid DNA hydrogel was the most cost-effective in that less DNA and Ag⁺ were used to produce the same amount of gel. The same approach was also used to detect and remove mercury ions from solutions *via* the formation of a T-Hg²⁺-T bridge^{126,215}.

At a later stage, studies were performed involving the preparation of DNA hydrogels having internal duplex and triplex structures^{139,189,192}. It was revealed that the formation of these internal structures in the hydrogels occurred in a pH-dependent responsive manner (Figure 16, C). More specifically, the duplex and triplex structures were found to cooperatively coexist (or not) in the hydrogel depending on the pH of the environment, with any change in pH resulting in an internal reversible hydrogel-solution (gel-sol) transition cycle with shape memory. Based on the above concept, Willner and coworkers were able to prepare a T-A*T triplex-based DNA hydrogel that specifically binds to the anticancer drug, coralyne¹³⁹. Not only did complexation of the drug to the system increase the hydrogel stiffness, but the pH-controlled delivery of the drug was also demonstrated.

The same pH-dependent behaviour was demonstrated in hydrogels that were prepared by crosslinking polymer chains^{133,207} or nanoparticles²²⁴ with C-rich ssDNA strands, where ssDNA strand's cytosine protonation led to the formation of an i-motif structure (Figure 16, D). The systems developed here showed a hydrogel to quasi-liquid transition with shape memory that was dependent on pH, attributable to the pH sensitive cytosine protonation/deprotonation nature. Although considered as interesting systems, Willner's research group was also inspired by G-quadruplexes^{132,203}. Here, the G-rich ssDNA strands grafted on polymers self-assembled into parallel or antiparallel quadruplex structures in the presence of potassium, lead or ammonium ions (Figure 16, E), being dependent only on this factor. A great advantage of this quadruplex system lies in its ability to complex with the iron-containing porphyrin, hemin, resulting in horseradish peroxidase-like catalytic activity¹³².

The versatility of these DNA structures has led to a new generation of multi-trigger responsive shape-memory hydrogels^{191,220}, where the cooperative actions of the different DNA structures involved are merged. The cooperative crosslinking interactions of all duplexes, i-motifs and G-quadruplexes will result in a hydrogel with a well-defined shape and memory (Figure 16, C-E). From the moment some of the interactions are ceased, the hydrogel will suffer a hydrogel to quasi-liquid transition, momentarily losing its shape but not its hydrogel nature. The hydrogel will never forget its initial shape because the duplexes, which are responsible for memory (unless denatured), will maintain the minimal conditions to avoid complete dissolution.

In a more recent and novel approach, the hybridization chain reaction (HCR) was used to functionalise surfaces with DNA hydrogels (Figure 16, F-G)¹⁴⁰. Two ssDNA hairpins, H_A and H_B, were grafted onto polymers resulting in P1 and P2, respectively. The two hairpins were designed such that

they included caged sequences that crosslinked H_A and H_B to each other in a layer-by-layer like fashion. Briefly, a gold surface was functionalised with a ssDNA molecule that was complementary to H_A . This induced H_A anchorage to the open and exposed complementary sequence on the gold surface, which in turn served as a toehold trigger to open of H_B . This chain of events resulted in the formation of a DNA hydrogel film with interfacial electron transfer properties. Upon the further introduction of G-quadruplexes to the system, electrocatalytic functions were also introduced. This approach was then implemented to produce DNA hydrogel microcapsules using calcium carbonate microparticles as a template¹⁴⁴. The developed microcapsules were used for the controlled delivery of drugs in the presence of the appropriate triggers, which resulted in a decrease in gel stiffness and thus an increase in the permeability of the microcapsule shell.

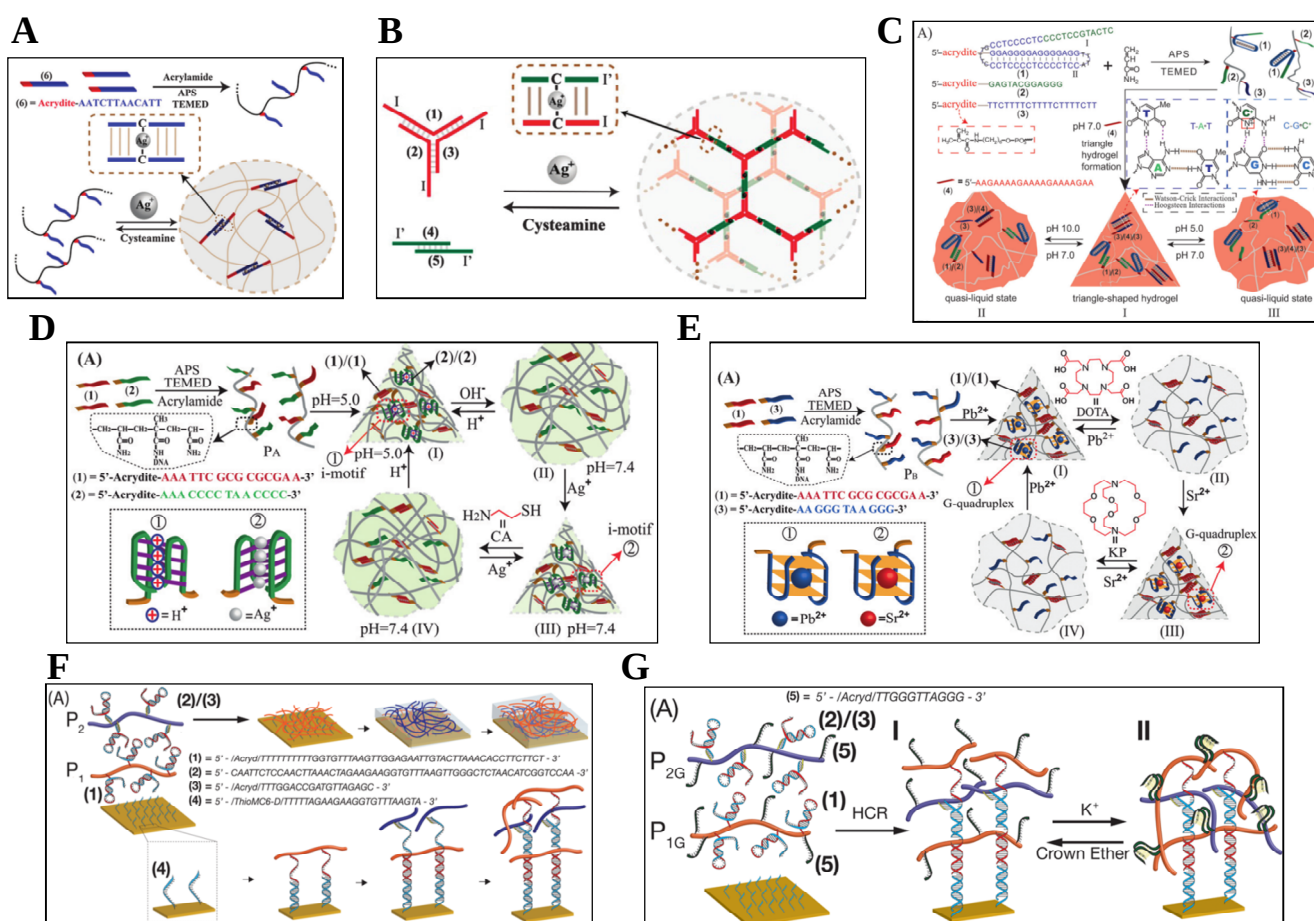


Figure 16 - The different DNA hydrogels produced by Willner's research group based on different DNA structures, namely duplexes, triplexes, i-motifs, G-quadruplexes and HCR. (A) ssDNA-grafted polymer hydrogel with a C-C mismatch stabilized by Ag^+ ¹³⁸. (B) Pure DNA hydrogel constructed by Y-DNA and a dsDNA linker that is stabilize Ag^+ ¹³⁸. Shape memory DNA hydrogels with (C) cooperative Watson-Crick and Hoosteen base pairs¹⁹², (D) Watson-Crick base pairs and pH-sensitive i-motifs²²⁰, and (E) Watson-Crick and ion sensitive G-quadruplexes²²⁰. (F) Deposition of the DNA hydrogel on a Au-coated glass surface using the hybridization chain reaction (HCR)¹⁴⁰. (G) Deposition by HCR of a G-quadruplex switchable DNA hydrogel on a Au-coated glass surface exhibiting controlled stiffness properties¹⁴⁰. Adapted/reprinted with permission from ref. 138,

As previously described, an alternative strategy for the production of DNA hydrogels involves the use of a third ssDNA strand that is complementary to the polymer-grafted ssDNA (Figure 14, A(ii))^{127,129,136,145,150,182,186–188,190,193,195,199,204,205,208,210–214,216–219,222,225}. The hydrogels here can undergo gel-sol transitions (Figure 14), either (i) as a result of their thermoreversible properties where DNA denaturation plays an important role, or (ii) by strand displacement *via* a DDR (DNA displacement reaction) process where a fourth ssDNA competes with a linker already present in the gel, responsible for gelation. Playing an important role in the preparation of these model hydrogel systems is the ability to rationally custom design DNA, since its function is dictated by its sequence. Many studies have exploited this and have used specific DNA sequences such as aptamers (DNA sequence with high affinity and specific antibody-like recognition and binding activity)^{226,227} and DNAzymes (DNA sequence or structure with enzyme-like catalytic activity)^{228,229} as linkers. Thus, the DDR process can be achieved either by a ssDNA strand that behaves as a toehold, by aptamer recognition of target molecular species or by DNAzyme catalytic activity towards a substrate in the presence of specific triggers.

Since 2004, Langrana's group has been preparing self-assembly DNA hydrogels by crosslinking ssDNA grafted polymers with a simple third linker ssDNA^{145,186–188,195,199,208,212,214}. In their work^{145,186}, the mechanical properties of various hydrogels were extensively evaluated at different crosslinking densities (Figure 17, A-E). The prepared hydrogels presented a transition from a liquid to a solid gel between 22.2% and 23.6% crosslinking density and were considered to be "solid" at higher crosslinking densities (>23.6%). As expected, the viscosity, elasticity, stiffness and strength of the gels were found to be dependent on crosslinking density and temperature. Moreover, the gels presented a transition in viscosity at 54°C, the melting temperature of the formed DNA duplex. Through their work, Langrana and coworkers also showed that the toehold-mediated displacement ssDNA was able to diffuse through the hydrogel and that this method allowed for the gel dissociation without any increase in temperature. As stiffness could be controlled by DDR^{195,208}, the hydrogels were modified through surface ligand functionalisation and then used as extracellular matrices to evaluate the influence of tunable stiffness on the growth, morphology and behaviour of fibroblasts and neural cells (Figure 17, F-O)^{188,199}. Not only were neurons shown to extend more primary dendrites and shorter axons on stiffer gels, but also an increase in neuronal and total cell number was observed on these gels. The proportion of neurons and astroglia combined in the total cell population however remained stable. The possibility of changing the crosslinking density without increasing the temperature was further assessed, as the cells in contact with the hydrogel were observed to react

to dynamic stiffness, that is, to the *in situ* decrease of hydrogel stiffness^{212,214}. The studies performed here were very deep, with many parameters and conditions taken into account. In case of interest, readers are referred to references 212 and 214 for further reading. Overall, the work demonstrates the importance of cell and extracellular matrix interactions on cell growth and morphology, providing guidelines for the production of biomaterial scaffolds for neural tissue engineering applications that have the right mechanical properties and a capacity to modulate tissue growth.

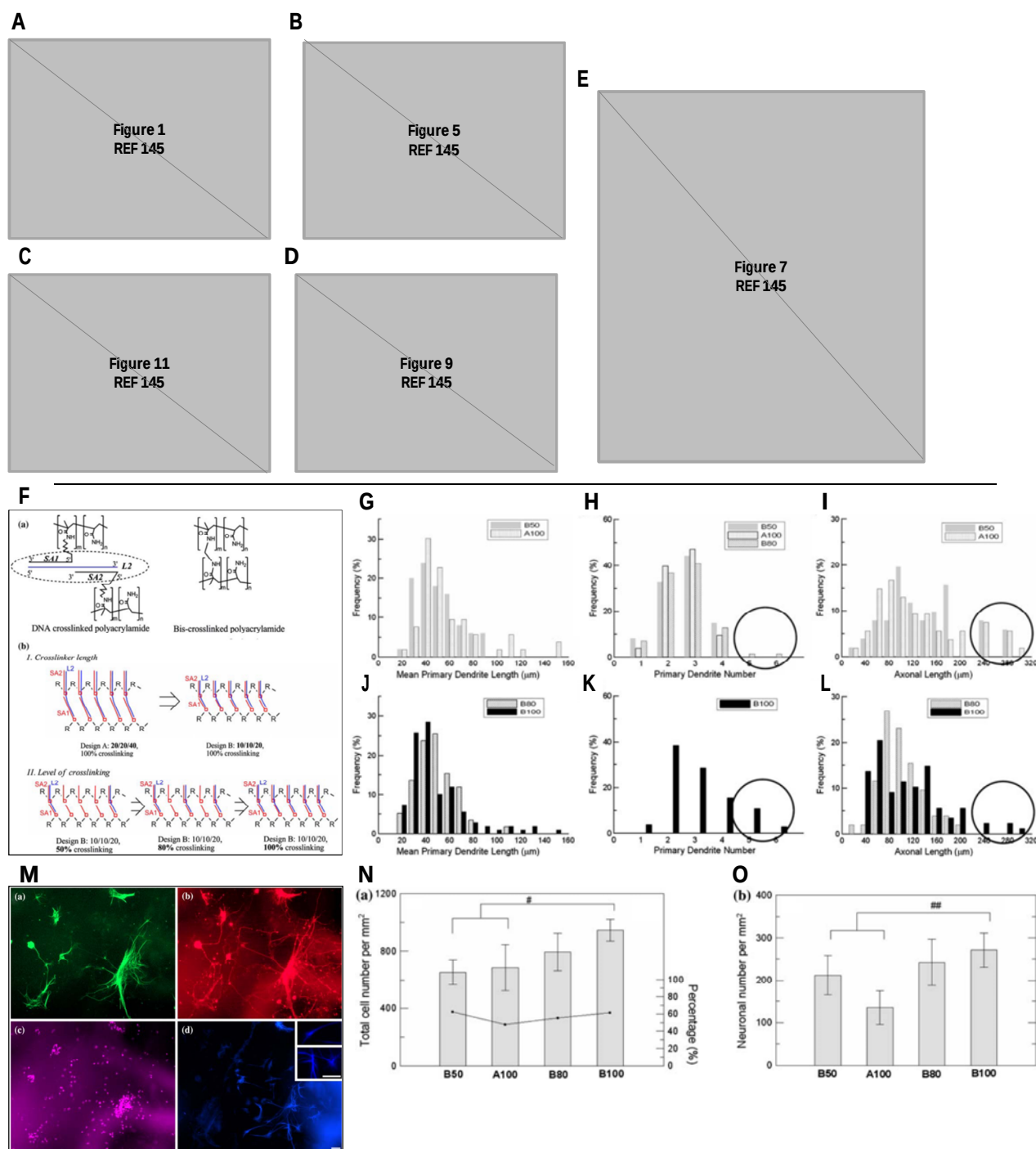


Figure 17 - Third DNA strand (linker) driven DNA hydrogels preparation. (A) Two ssDNA-grafted (SA1 and SA2) PA polymer chains crosslinked using a third ssDNA (L2)¹⁴⁵. (B) DNA hydrogel viscosity as a function of

crosslinker densities¹⁴⁵. (C) Dissociation of the DNA hydrogel network by displacement DNA strand (R1)¹⁴⁵. (D) Elastic modulus as a function of crosslinker density¹⁴⁵. (E) Elapsed-time comparison of the behaviour of the DNA hydrogel in the presence of either a solution of removal DNA (top) or a solution of TE buffer (bottom). The distribution density of the fluorescent spheres is indicated in each respective schematic representation¹⁴⁵. (F) Chemical structure of DNA-PA gels, with SA1 and SA2 as the grafted ssDNA strands and L2 as the linker. L2 is complementary to both SA1 and SA2. SA1, SA2 and L2 can have different lengths and crosslinking densities (50%, 80%, and 100%)¹⁹⁹. Frequency plots of mean primary dendrite length (G, J), primary dendrite number (H, K) and axonal length per neuron (I, L) on two different DNA hydrogels¹⁹⁹. (M) Attachment and growth of rat spinal cord cells on DNA crosslinked gels. MAP2 immunostaining for dendrite and soma (green), Tau-1 immunostaining for axon (red), DAPI staining for intact nuclei (purple) and GFAP immuno-staining for mature astroglia (blue)¹⁹⁹. Changes in total cell numbers (N) and neuronal cell numbers (O) in response to the stiffness of the DNA crosslinked gels¹⁹⁹. Adapted/reprinted with permission from ref. 199, Copyright © 2008 Springer Nature.

Still, following the same theme of extracellular matrix production, Li *et al.* were able to prepare a supramolecular DNA-based hydrogel using a rapid "printing" method²⁰⁴. The process developed involved the alternative deposition of a ssDNA-polypeptide (bio-ink A) and a dsDNA linker (bio-ink B) in a programmed position resulting in a self-supported and free-standing tridimensional polypeptide-DNA hydrogel (Figure 18, A-C). The alternating depositions showed no boundaries between layers due to the self-healing properties of the hydrogel. Due to its biocompatibility, permeability, and biodegradability by nucleases and proteases under physiological conditions, the prepared hydrogel was considered to be an ideal biomaterial for the *in situ* 3D printing of cells. The hydrogel provided physical support for the cells and allowed for the diffusion of nutrients, both essential for cell viability. More recently, Li and coworkers prepared a similar polypeptide-DNA hydrogel, where its mechanical properties were studied as a function of the sticky ends length or the presence of mismatches in the dsDNA crosslinker (Figure 18, D-H)²²². Without changing the crosslink density but tuning the duplex stability, they were able to change the rheological properties of the hydrogel. Conformational changes in the polypeptide from random coil to α -helix (and *vice-versa*) as a result of changing the environmental pH, were also found to influence the hydrogel properties^{222,230}. Based on the results obtained thus far for these supramolecular polypeptide-DNA hydrogels, it is likely that future studies will focus on mechanical-induced cell differentiation *in situ* and other potential tissue engineering applications.

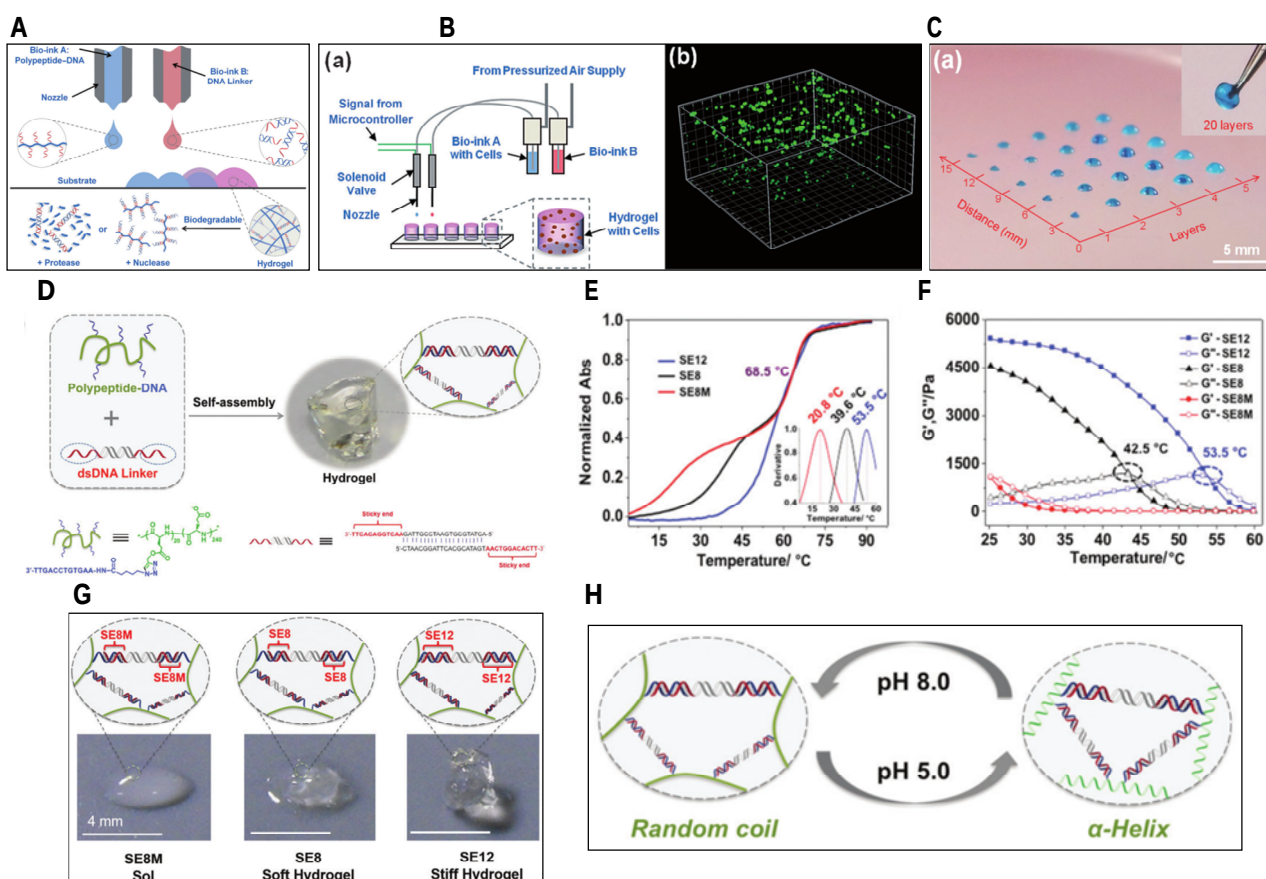


Figure 18 - Self-assembled DNA-polypeptide hydrogel with tunable degradation and conformation-dependent swelling behaviour. (A) 3D bioprinting of tridimensional DNA hydrogel structures by crosslinking polypeptide-DNA (bio-ink A) and DNA linker (bio-ink B). The formed hydrogels are responsive to proteases and nucleases²⁰⁴. (B) (a) AtT-20 cell-printing process based on ink-jet techniques and (b) its 3D stacking based on fluorescein diacetate (FDA) green fluorescence²⁰⁴. (C) Array of printed droplets with an increasing number of layers²⁰⁴. (D) Supramolecular hydrogel preparation by DNA hybridization, where the two complementary polypeptide-DNA and dsDNA linker components are mixed²²². (E) Melting points of the DNA linker with different sticky ends lengths determined by UV-thermal spectra from 4°C - 90°C and the respective first derivative of each curve from 4°C - 60°C (inset curve)²²². (F) Gel-sol transition process characterized by a temperature sweep from 25°C - 60°C with a fixed frequency of 1 Hz, a strain of 1%, at 2°C/min²²². (G) Tuning the mechanical properties of the hydrogels by increasing or decreasing the sticky ends lengths with 8 bp (SE8) or 12 bp (SE12) or inserting a mismatch (8 bp mismatch, SE8M) without changing the crosslinking point density and the network topology²²². (H) Tuning the mechanical properties of the hydrogels by changing the environmental pH, leading to a conformational transition of the secondary structure of the polypeptide backbone between random coil and α -helix²²². Adapted/reprinted with permission from ref. 204, Copyright © 2015 by John Wiley & Sons, Inc. and ref. 222, Copyright © 2017 The Royal Society of Chemistry.

Hybrid hydrogels can also be prepared by combining independent networks made up of different materials. An interpenetrating DNA and phenylalanine-functionalised carboxymethyl cellulose/cucurbit[8]uril (CMC-phe/CB[8]) double network hydrogel was prepared by Li *et al.*²³¹ using a specific recognition self-assembly "one-pot" method (Figure 19). Here, recognition between a Y-DNA structure and a linear DNA linker occurs leading to their self-assembly *via* base pair specific recognition (a method commonly used to produce pure hydrogels; as will be explained later in this

review). Parallel to this, CB[8] recognizes the phenylalanine groups in CMC-phe as a result of host-guest interactions leading to the formation of a physically crosslinked interpenetrating network. Separately, each crosslinked system has the ability to form a hydrogel with distinct mechanical properties. However, combining the four components together *via* a "one-pot" method results in the formation of a double interpenetrating supramolecular hydrogel system with different mechanical properties (*i.e.* flexibility and strength), where one hydrogel network compensates the other²³². Interestingly, the complex polymeric network could be selectively degraded by nucleases (in the case of DNA) or by proteases (in the case of cellulose) such that a single network hydrogel alone is obtained²³¹. This new and soft material has great potential as a scaffold system for tissue engineering purposes.

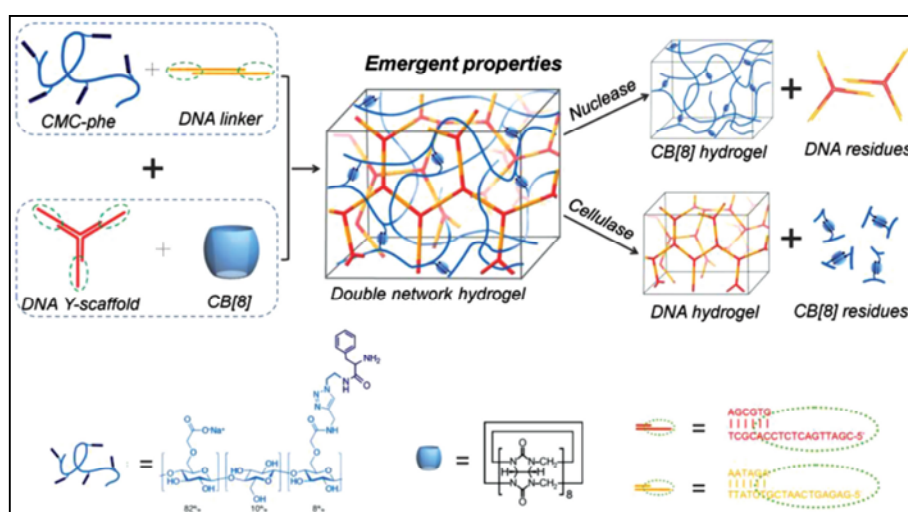


Figure 19 - Formation process of double network hydrogels through supramolecular interactions in a "one-pot-method". Phe-modified CMC forms complexes with CB[8], while the second network is crosslinked by hybridization of DNA sticky ends. The resulting double network hydrogel possesses the combined properties of each single hydrogel network, and presents stimuli-responsive behaviour to specific enzymes.²³¹ Adapted/reprinted with permission from ref. 231, Copyright © 2015 by John Wiley & Sons, Inc.

Other physically crosslinked hybrid DNA-based hydrogels

In the previous section, several works were described where hydrogel formation relied on the DNA base pair recognition process and, thus, on the establishment of specific hydrogen bonds. However, hybrid DNA-based hydrogels can be crosslinked through other types of physical interactions (non-covalent bonds), such as *van der Waals* forces, ionic bonds, π - π stacking, hydrophobic interactions or still hydrogen bonds but other than those involved in DNA hybridization. In this scope, hydrogels containing DNA were prepared in combination with a great variety of molecules/materials, like for example cationic surfactants/lysozyme¹⁴⁷, poly(vinylalcohol) (PVA)²³³, poly(phenylenevinylene) (PPV)¹⁴⁹, graphene oxide (GO) sheets¹²⁵, single-walled carbon nanotubes

(SWNTs)¹³⁰, hectorite (clay)²³⁴, carboxymethyl cellulose (CMC)²³¹ and tannic acid (TA)²³⁵. Often, hydrogel formation is a result of the simultaneously action of different kinds of physical interactions.

For example, DNA hydrogels produced by mixing DNA with either the cetyltrimethylammonium bromide (CTAB) surfactant or lysozyme¹⁴⁷ are based on the electrostatic interactions between these oppositely charged molecules. The hydrogels prepared here were observed to form stable gel particles as a result of electrostatic interactions and they were reported to display a swelling/deswelling behaviour in buffer solution. The DNA was also readily released from the gel particles, a feature that may have future in gene delivery applications.

Also, a fluorescent DNA hydrogel was synthesized by *in situ* polymerization of phenylenevinylene (PV)¹⁴⁹ using DNA as a template (Figure 20). Briefly, the self-assembled DNA hydrogel was prepared by electrostatic interactions between DNA strands and the positively charged sulfonium precursor poly(phenylenevinylene) (SP-PPV) polymer. A critical crosslinker concentration was detected during hydrogel production, with values below this limit resulting in an unstable hydrogel. The crosslinking density of the hydrogel was also found to affect its swelling degree. More specifically, a lower swelling degree was observed in the hydrogel at higher crosslinking density. Circular dichroism studies showed that the hydrogel synthesis conditions did not affect the DNA's B-conformation. The hydrogel was also observed to be resistant to degradation as it was unaffected by nuclease digestion, heat and ultrasonic forces. The uniqueness of this DNA hydrogel lies in its strong intrinsic fluorescence properties. After loading the hydrogel with anti-hypertensive nifedipine hydrochloride, a drug capable of totally quenching the gel's intrinsic fluorescence properties, drug release culminated in the restoration of the gel's initial fluorescence intensity.

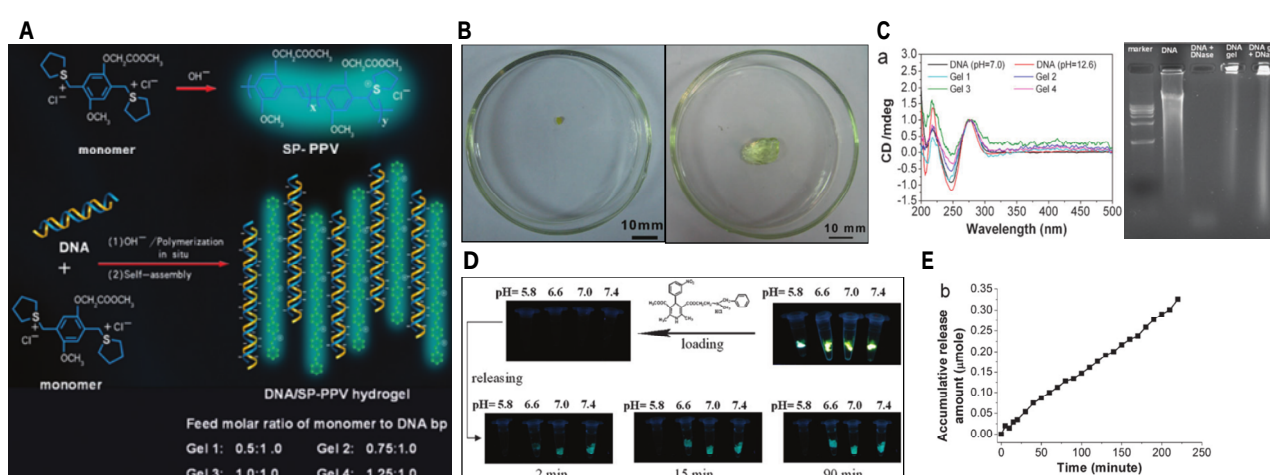


Figure 20 - Ionic interactions driven DNA hydrogel with intrinsic fluorescence. (A) Synthetic reaction of PPV derivative from monomer and preparation of DNA/SP-PPV hydrogels. Monomer: DNA bp ratios of 0.5, 0.75, 1.0 and 1.25 resulted in the formation of Gel 1, Gel 2, Gel 3 and Gel 4, respectively. (B) DNA/SP-PPV hydrogels in a dried form and swollen in water for 12h. (C) Circular dichroism of wet DNA/SP-PPV hydrogels (normalized with

respect to the intensity of the band at 275 nm) and gel electrophoresis of DNase I digestion on DNA and Gel 2. (D) Monitorization of the drug release from Gel 2 by observing changes of emission intensity of the gel after immersing it into 50mM PBS (pH 5.8, 6.6, 7.0 and 7.4) at different time points (2, 15 and 90 min). (E) Controlled release profile of drug from Gel 2 at medium pH of 6.6 (no burst release was observed)¹⁴⁹. Adapted/reprinted with permission from ref. 149, Copyright © 2009 The Royal Society of Chemistry.

A very interesting work was conducted by Xu *et al.*¹²⁵, where multifunctional hydrogels were synthesized through the three-dimensional self-assembly of graphene oxide (GO) sheets and DNA (Figure 21, A-B). Here, π - π stacking interactions between DNA and the GO component governed the assembly of the hydrogel. A key parameter in this process was the denaturation of dsDNA upon heating, which led to the exposure of π electron clouds and their subsequent bridging between adjacent GO sheets *via* strong non-covalent interactions. These gels were capable of self-healing after a heat-cool process. The same type of interactions (π - π stacking) were also established between DNA and single walled carbon nanotubes (SWNT) in the case of the DNA-SWNT hybrid hydrogel developed by Cheng and coworkers¹³⁰. Two components were used in the preparation of this gel, namely, linear dsDNA with sticky ends (the linear units) and DNA-carbon nanotubes (the crosslinker units) which also had DNA sticky ends exposed. For pH values below 6.3, intermolecular i-motif structures were formed between the existent sticky domains present in the hydrogel components (Figure 21, C-D). These hydrogels were pH responsive (changed for a solution state upon a pH increase) and strength tunable within certain limits.

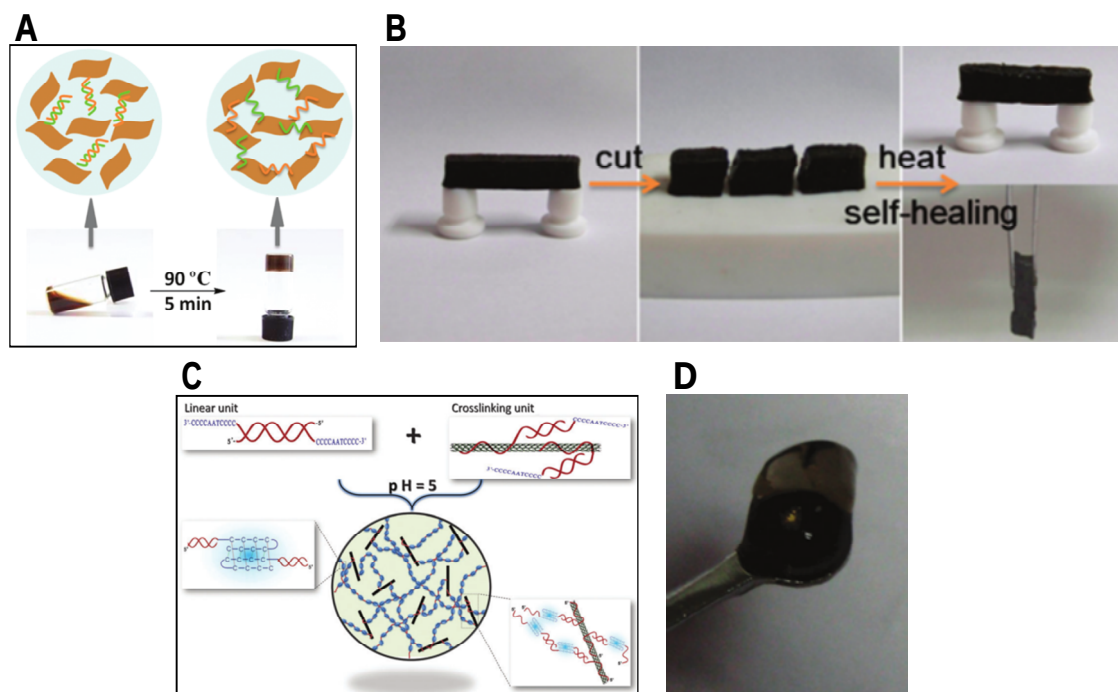


Figure 21 - π - π interactions driven DNA hydrogels with self-healing properties. (A) Procedure for preparing three-dimensional self-assembled GO/DNA hydrogels and the gelation mechanism¹²⁵. (B) Self-healing process where a gel block was first cut into pieces and then after a heat-cool process the pieces re-adhered to each other¹²⁵. (C) The formation of a DNA-SWNT hybrid hydrogel¹³⁰. (D) Optical image of the DNA-SWNT hybrid hydrogel prepared at pH 5.0¹³⁰. Adapted/reprinted with permission from ref. 125, Copyright © 2010 American Chemical Society and ref. 130, Copyright © 2011 The Royal Society of Chemistry.

A unique study by Shin *et al.*²³⁵ revealed the formation of a DNA hydrogel based on interactions with the phosphate groups in the DNA backbone (Figure 22). Tannic acid (TA), a plant-derived polyphenol, was used as a "molecular glue" where it interacted with the DNA phosphodiester bonds through hydrogen bonds to form a TNA hydrogel (TA+DNA). Due to its intrinsic hydrolyzable ester bonds and adhesive catechol/pyrogallol groups, TA conferred the hydrogel with both degradability (DNA release was confirmed by gel electrophoresis) and adhesive (force exerted was verified by a universal testing machine) properties. An interesting fact was that gelation only occurred when using a [DNA base pair]/[TA] ratio of 1.3 or lower, resulting in a more robust hydrogel. At a ratio of 2.6, gelation did not occur. This data is in contrast to that reported in previous studies, where a higher amount of crosslinker usually results in gelation and the hydrogel presents stronger mechanical properties. These TNA hydrogel revealed as well, thermosensitivity, where elasticity (G') and viscosity (G'') decreased with temperature due to dsDNA denaturation. The hypothesis presented for the observed gelation properties of the TNA hydrogel was the oxidation of TA above a pH of 7. More specifically, with increasing amounts of TA, the pH also increased. Thus, at a higher TA concentration, the pH got closer to 7 and promoted TA's own oxidation thereby preventing hydrogel formation. As the TNA hydrogel was formed by hydrogen bonds, the authors were curious to see if the gel would exhibit the same thermoreversible gel-sol transitions as DNA hydrogels prepared by

hybridization. This was in fact confirmed. Adhesiveness was shown to be an important feature for potential biomedical applications, namely as hemostatic agents due to the polyphosphate backbone and for subcutaneous tissue adhesion. In the case of their use as hemostatic agents, the ability of the TNA hydrogels to stop bleeding was evaluated. Hemostasis times were impressively decreased when using TNA hydrogels. In the negative control, hemostasis was complete after 133 s. Hemostasis was completed for DNA and TA after 97s and 100s, respectively. Regarding the TNA hydrogels, the time was 53s, approximately 2.5x less time relative to the negative control²³⁵. Overall, this hydrogel shows very good qualities to evolve for future biomedical applications.

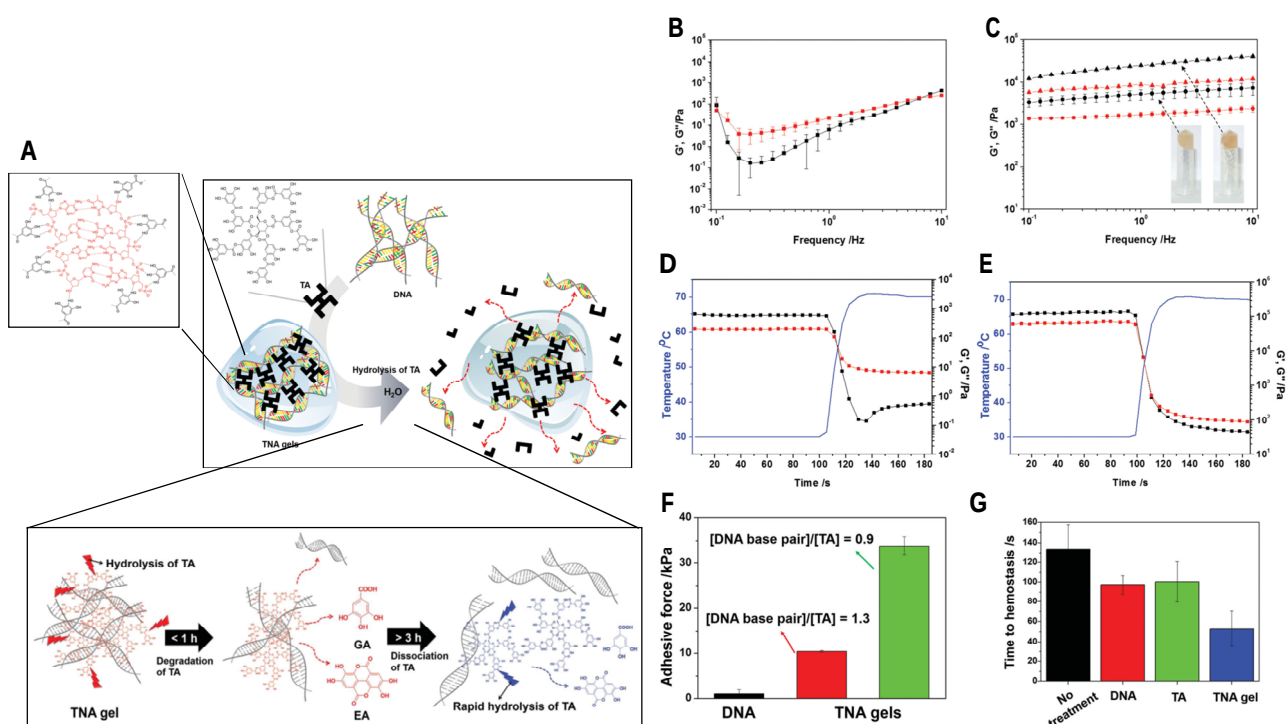


Figure 22 - Hydrogen bond driven DNA hydrogel with tannic acid. (A) Formation and degradation of TNA gels. (B) Mechanical properties of a TNA gel at a [DNAbp]/[TA] ratio of 2.6, namely its elastic modulus (G' , black) and its viscous modulus (G'' , red). (C) Mechanical properties of TNA gels at [DNAbp]/[TA] ratios of 1.3 (circle) and 0.9 (triangle). The inset photos show the gelation of each TNA gel. (D) The gel-to-sol transition behaviour of a TNA gel at a [DNAbp]/[TA] ratio of 1.3. (E) The gel-to-sol transition behaviour of a TNA gel at a [DNAbp]/[TA] ratio of 0.9, taking into account temperature (blue), elastic modulus (G' , black) and viscous modulus (G'' , red). (F) Increase in adhesion force of DNA alone and in TNA gels at different [DNAbp]/[TA] ratios. (G) Time required for complete hemostasis in a bleeding mouse liver, without treatment or with treatment using a DNA solution only, a TA solution only and a TNA gel.²³⁵ Adapted/reprinted with permission from ref. 235, Copyright © 2015 by John Wiley & Sons, Inc.

2.2. Pure DNA-based hydrogels

Pure DNA hydrogels are composed of DNA alone, with no other molecules involved in their final structure. In many situations, the hydrogels are composed of dsDNA chains linked by stable covalent bonds. Frequently, pure DNA-based hydrogels also rely on the establishment of physical interactions, like the specific hydrogen bonds involved in DNA base pair recognition, or other non-covalent bonds like those mentioned before for the hybrid hydrogels. Also here, physically crosslinked hydrogels can be disrupted by simple environmental changes, such as temperature, pH, ionic strength, etc.¹². Thus, they are expected to be more susceptible to degradation and mechanically less resistant¹⁶².

Pure DNA-based hydrogels obtained by enzymatic ligation

Enzymatic ligation is a biochemical approach used to connect different branched DNA structures to form hydrogels. This strategy commonly uses the enzymatic activity of T4 DNA ligase which has the ability to join hybridized sticky ends to close a nick site or to connect blunt ends in dsDNA strands (Figure 23) by covalently connecting adjacent nucleotides through a phosphodiester bond³⁵.

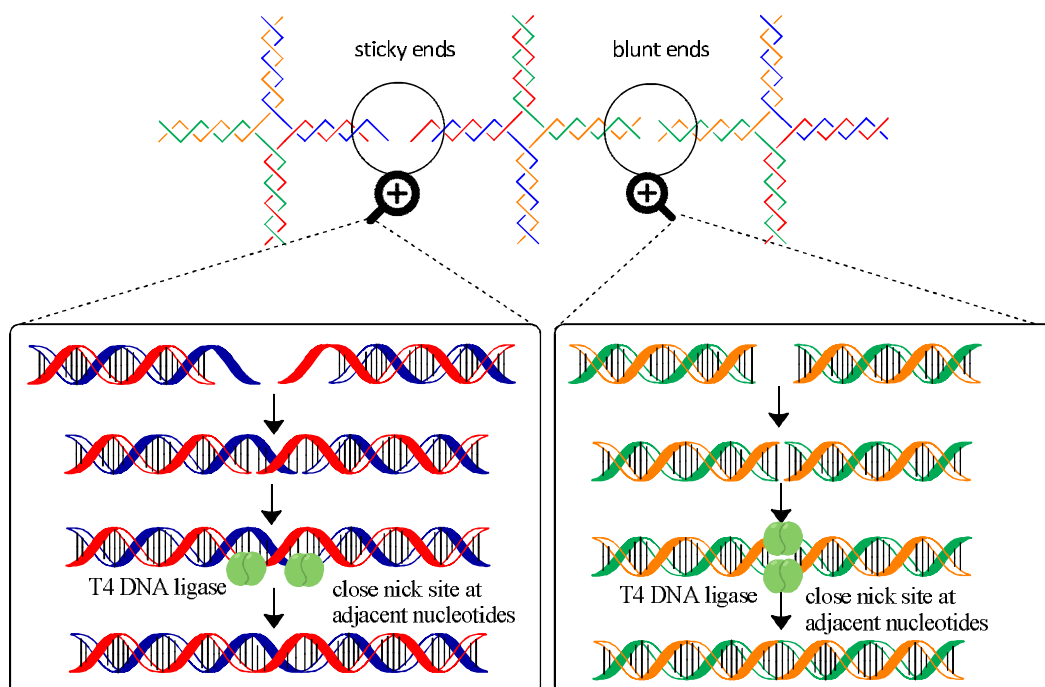


Figure 23 - T4 DNA ligase in action, joining two DNA molecules with either sticky or blunt ends in order to achieve the hydrogels tridimensional network.

This very simple procedure was used in 2006 by Um *et al.*¹⁴⁶, and it has since been adopted by many others^{124,142,146,236–245} to produce hydrogels for different applications. The advantage of this process is that it can be accomplished under physiological conditions, allowing for *in situ* encapsulation without side reactions or inactivation/denaturation of biomolecules. Also, no extra crosslinkers are needed, decreasing the chance of toxicity effects in case of its use in biomedical applications.

The first pure bulk DNA hydrogel created through this process involved three different shaped DNA structures, namely X-, Y- and T-DNA¹⁴⁶. In each case, the DNA structure was prepared such that each of its arms possessed sticky end complementary palindromic sequences that would be able to hybridize and ligate with each other (Figure 24, A-C). Hydrogel preparation thus occurred by covalently binding these DNA structures using T4 DNA ligase. Several hydrogels were obtained in this manner, each with distinct swelling and mechanical properties. The swelling degree was shown to be dependent on the initial DNA structure used ($X > Y > T$) and was higher in hydrogels prepared with more DNA. The stiffness of each hydrogel, reflected by its tensile modulus, was in the order of $X > T > Y$. And finally, the tensile strength of each hydrogel, where gel elongation until breakage point was assessed, was in the order of $T > Y > X$. Based on these results, the X-DNA hydrogel presented a higher resistance to deformation (*i.e.* high tensile modulus, elasticity) probably due to a higher crosslinking density. It also displayed less resistance to return to its original shape, being more prone to breakage (*i.e.* low tensile strength, breaking point). The prepared hydrogels also presented shape-memory upon consecutive dehydration-hydration cycles. Along with these properties, the hydrogels were also found to display controllable biodegradation ($Y > T > X$). This was found to be an advantage for the controlled release of drugs such as camptothecin (CPT) and insulin after their *in situ* encapsulation. Finally, the DNA hydrogels prepared here under physiological conditions were also found to be effective, non-toxic systems for the encapsulation of live cells, showing great potential as biomaterials for tissue engineering and 3D culture.

In this scope, a very innovative material was prepared by Park and coworkers^{124,239}. Through the incorporation of linear plasmid DNA (pDNA) crosslinked with X-DNA by T4 DNA ligase (Figure 24, D-F), a novel cell-free protein production platform (P-gel) was obtained in the presence of cell lysates. This system was found to be 300 times more efficient than the current protein synthesis systems. The amount of X-DNA used was critical for gene expression, since a higher content of X-DNA resulted in less gene expression. Probably what happened was that at higher X-DNA concentrations the gel gets stiffer, more like a solid than a hydrogel, not allowing for the easy diffusion of enzymes and precursors. Using this platform, 16 different proteins were successfully synthesized.

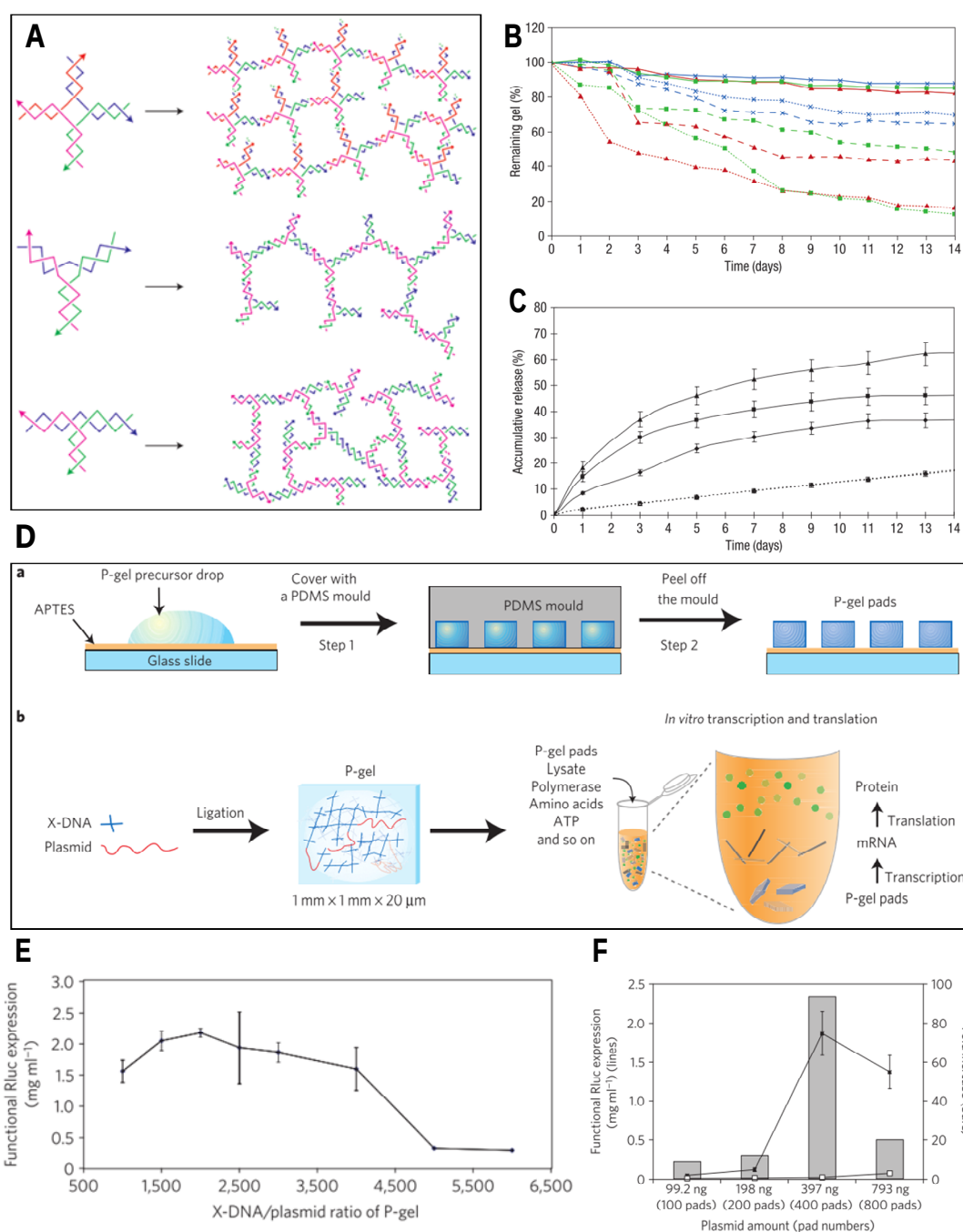


Figure 24 - Enzymatic ligation driven DNA hydrogel, with branched DNA molecules and/or branched DNA molecules, for cell-free protein synthesis. (A) Branched DNA structures (X-, Y- and T-DNA) that after T4 DNA ligase activity resulted in crosslinked DNA hydrogels with different properties¹⁴⁶. (B) DNA hydrogel degradation profile based on the percentage mass of DNA remaining in the system as a function of time (dotted lines - empty gels, dashed lines - insulin-loaded, solid lines - CPT loaded, blue symbols - X-gel, red symbols - Y-gel, green symbols - T-gel)¹⁴⁶. (C) Insulin and CPT release profiles determined by the ELISA enzyme immunoassay kit and absorbance at 370 nm (solid lines - insulin, dashed line - CPT, diamond symbols - X-gel, triangle symbols - Y-gel and square symbols - T-gel)¹⁴⁶. (D) Formation of P-gel micropads (top), starting off with a drop containing the precursor X-DNA, genes and T4 DNA ligase that are all first confined and then released from a poly(dimethylsiloxane) (PDMS) mold. The gelation process that occurs through enzymatic crosslinking, followed by cell-free expression with P-gel pads (bottom)¹²⁴. (E) Effect of the X-DNA crosslinker/plasmid ratio of P-gel on functional RLuc expression¹²⁴. (F) Effect of the total gene amounts on expression, determined by varying the number of P-gel micropads in the reaction (filled squares). The same amounts of the plasmid were used in solution phase synthesis (SPS) as the established control reactions (open squares)¹²⁴. Adapted/reprinted with permission from ref. 124, Copyright © 2009. Springer Nature and ref. 146, Copyright © 2006 Springer Nature.

Nishikawa and coworkers²⁴¹ were able to incorporate various attractive features into X-DNA-based hydrogel systems, ranging from chemical and physical features to biodegradability and controlled release properties, as well as the incorporation of immunostimulatory CpG motifs^{246,247}. The intercalation of doxorubicin into the CpG DNA hydrogel conferred it with more resistance to degradation. The simultaneous drug sustained delivery and immunostimulation was found to have synergistic effects, leading to a higher antiproliferation of cancer cells and to a decrease in tumour size when compared with the CpC DNA hydrogel, the CpG free DNA hydrogel and the drug alone.

The evolution from bulk to micro- and nanoscale materials is inevitable if biomedical *in vitro* and *in vivo* applications are desired. At the microscale, very interesting works on enzyme-catalyzed DNA hydrogels have been published such as the study of Kahn and colleagues that produced microgels for cell-free protein synthesis (Figure 25, A)²⁴³. These researchers developed a protein producing gel (P-gel) through the covalent linking of genes which was also able of displaying the produced protein by incorporating a molecular linker. Another interesting work was presented by Kim *et al.* that prepared DNA-based microgels for protein delivery and single cell encapsulations (Figure 25, B)²⁴⁴. They used a droplet generating microfluidic device to achieve an appropriate microscale format for DNA hydrogel production. At the nanoscale, enzyme catalyzed-nanohydrogels were prepared by enclosing X-DNA and T4 DNA ligase in a lipid vesicle²⁴⁰. The content and nature of the lipid component was found to influence the success and size of the prepared nanogels, where neutral and low amounts of lipid were essential for nanogel production. Lipid removal was possible when using a surfactant or phospholipase. Encapsulation and release of ovalbumin (OVA) showed that crosslinked nanogels released their content in a very sustained and controlled manner when compared with uncrosslinked particles. This new lipid vesicle methodology was the turning point for the development of synthetic nucleus-like systems (Figure 25, C-D)^{236,237,245}. As mimics of the cell nucleus, the nanosystems are composed of a lipid vesicle and DNA core which evoke the nuclear membrane and chromatin, respectively. Combined with a couple of transcription-translation systems, the gel system has proved to be effective as an *in vitro* expression platform for a variety of protein groups^{236,237,245}.

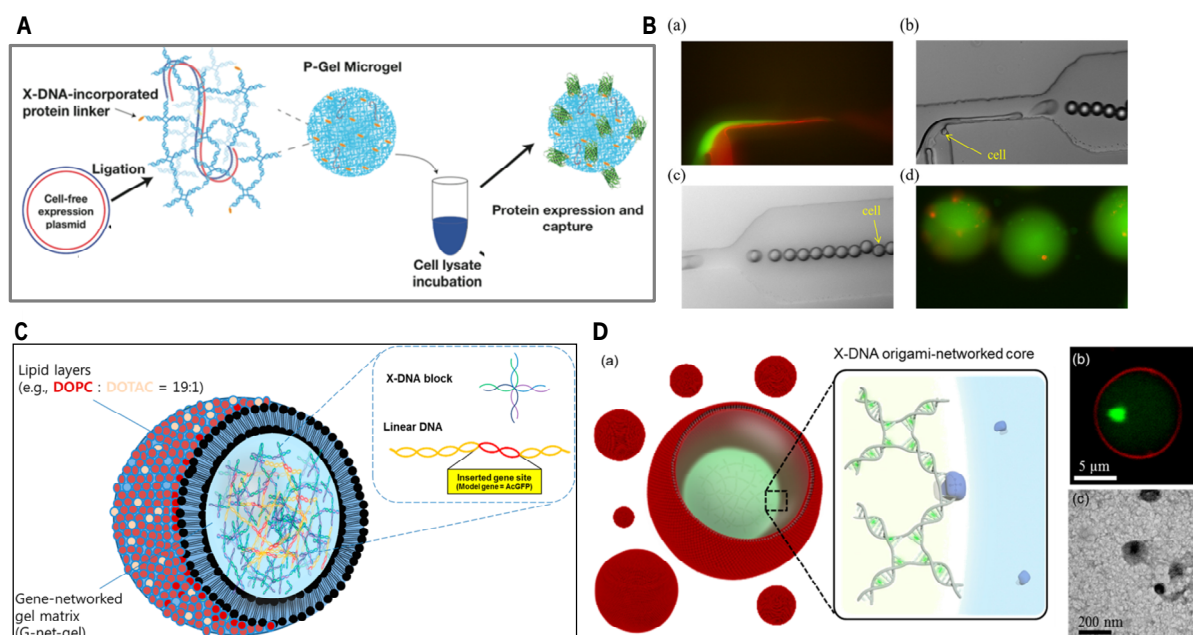


Figure 25 - Enzymatic ligation driven DNA hydrogel for cell-free protein synthesis, for single cell encapsulation and for the construction of a lipid-hydrogel nucleus-like structure. (A) The P-gel microgel is formed through the covalent linking of genes into a X-DNA network. The molecular linker for protein display is attached to the terminal end of X-DNA in the P-gel format²⁴³. (B) (a) Fluorescence image of the microchannel junction where DNA and cell solutions, stained with green and red fluorescent dyes, respectively (merged). (b) and (c) Single cell encapsulation into a DNA hydrogel microdroplet. (d) Fluorescence image of cells (red) encapsulated within DNA hydrogel microspheres (green)²⁴⁴. (C) Synthesis of a synthetic nucleus system based on the combination of a G-net-gel as a core-nucleus and an outer lipid membrane²⁴⁵. (D) (a) A T4 DNA ligase (blue) crosslinked X-DNA origami (gray/green) and core-supported by a lipid strata structure (red). (b, c) External morphologies shown using confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM), respectively²³⁷. Adapted/reprinted with permission from ref. 237, Copyright © 2015 American Chemical Society, ref. 243, Copyright © 2016 American Chemical Society, ref. 244, Copyright © 2016 AIP Publishing and ref. 245, Copyright © 2012 American Chemical Society.

Pure DNA-based hydrogels crosslinked by specific base pair recognition

Hydrogen bonding between two DNA strands is inherent to almost all DNA hydrogels. The most common procedure to promote ssDNA molecule and/or sticky end hybridization with the purpose of obtaining a pure DNA hydrogels is by means of temperature change^{128,142,143,157,162,248–254}. Here, DNA hydrogel formation occurs after first heating DNA above its T_m and then slowly cooling it to room temperature.

At high concentrations, long DNA chains of natural origin are unable to find and completely hybridize with one corresponding DNA chain alone, but rather they will randomly hybridize with several DNA chains to form a network²³. Hydrogels of this nature have been prepared along side EGDE crosslinked DNA hydrogels, allowing for the comparison of their mechanical properties^{157,162}. For example, Karacan *et al.*¹⁶² showed that physical DNA gels exhibit a high modulus of elasticity (G)

and that the hydrogen bonds were not enough to hold its tridimensional network during swelling. This is in contrast to what happens with chemical hydrogels. It was concluded that the swelling of both physical and chemical hydrogels is likely to be controlled by the ion and DNA concentrations in solution.

On the other hand, rationally designed DNA sequences and structures will allow for the formation of hydrogels with well-organized networks and controllable mesh/pore sizes. A very common strategy adopted to produce such hydrogels is the use of ssDNA as a starting point that will be assembled into branched units (e.g. X-, Y- or T-DNA). These units in turn will form links between each other *via* their sticky ends or *via* a DNA linker with sticky ends that after a heat-cooling cycle will promote DNA hybridization^{128,142,143,250–254}.

Lui and coworkers have worked extensively with these DNA-based physical hydrogels over the years^{128,143,250,254}, publishing interesting research that is worth consulting²⁵⁵. Y-DNA, for example, was reported to self-assemble with a linker¹²⁸ through a process promoted by a heat-cooling cycle (Figure 26, A-G). A sol-gel transition was revealed in the hydrogel produced here, which was confirmed by rheological tests in which the shear-storage modulus (elasticity, G') was noticeably higher than the shear-loss modulus (viscosity, G''). Hydrogel formation and mechanical strength were found to be dependent on the molar ratio between Y-DNA, linker and DNA concentration. The length of the sticky ends also influenced hydrogel mechanical strength, with small sticky ends made up of 4 bp revealing a low G'/G'' ratio and thus less mechanical strength, while 8 bp and 12 bp sticky ends showed higher G'/G'' values. Being entirely composed of DNA, the hydrogel was observed to be responsive to biodegradation by DNases and denaturation *via* temperature changes. Moreover, degradation was found to be highly specific, which was mainly attributable to the rational design of the DNA strands where specific sequences containing restriction sites were included in the linker. The application of this DNA hydrogel as a cover to hold and then release single cells in PDMS microwells was also explored (Figure 26, H-I)²⁵⁰. It was found that exposure of the hydrogel to specific restriction enzymes allowed for the controlled release of individual cells. Moreover, the approach used proved to be an effective, non-toxic and biodegradable strategy to control, manipulate and study individual cells and their communication. More recently, pH-sensitive C-rich DNA sequences constituting i-motif nanomotors were inserted into the linear linker component of this DNA hydrogel¹⁴³. In doing so, the hydrogel could undergo a pH-dependent conformational transition, allowing for the *in situ* reversible tuning of the mechanical properties of the gel (Figure 26, J). At a pH value of 6.3 or lower, the i-motifs were found to be active in a four-stranded structure. As the pH increased, the cytosines were no longer protonated and the i-motifs disassembled into single-stranded structures. This transition led to a change in the distance between that different crosslinking points in the Y-DNA structure, as well as the rigidity of the network. Combined, this work

has allowed for the evaluation of stem cell differentiation and tissue engineering under *in situ* dynamic mechanical properties. More recently, Lui and his research team have worked on developing a strategy to construct tissue-like complex structures using cell-laden DNA hydrogel bricks (Figure 26, K)²⁵⁴. Briefly, hydrogel bricks encapsulating different cell types were able to "glue" to each other due to the self-healing properties (due to DNA sequence complementarity) of the hydrogel. With this, the formation of different and complex tridimensional hydrogel constructions was made possible, permitting cell migration in 3D. Also, in this case, temperature showed to affect the viscoelastic properties of the hydrogels. This new technology holds great potential for artificial tissue fabrication as it appears convenient, effective and reliable for cell manipulation.

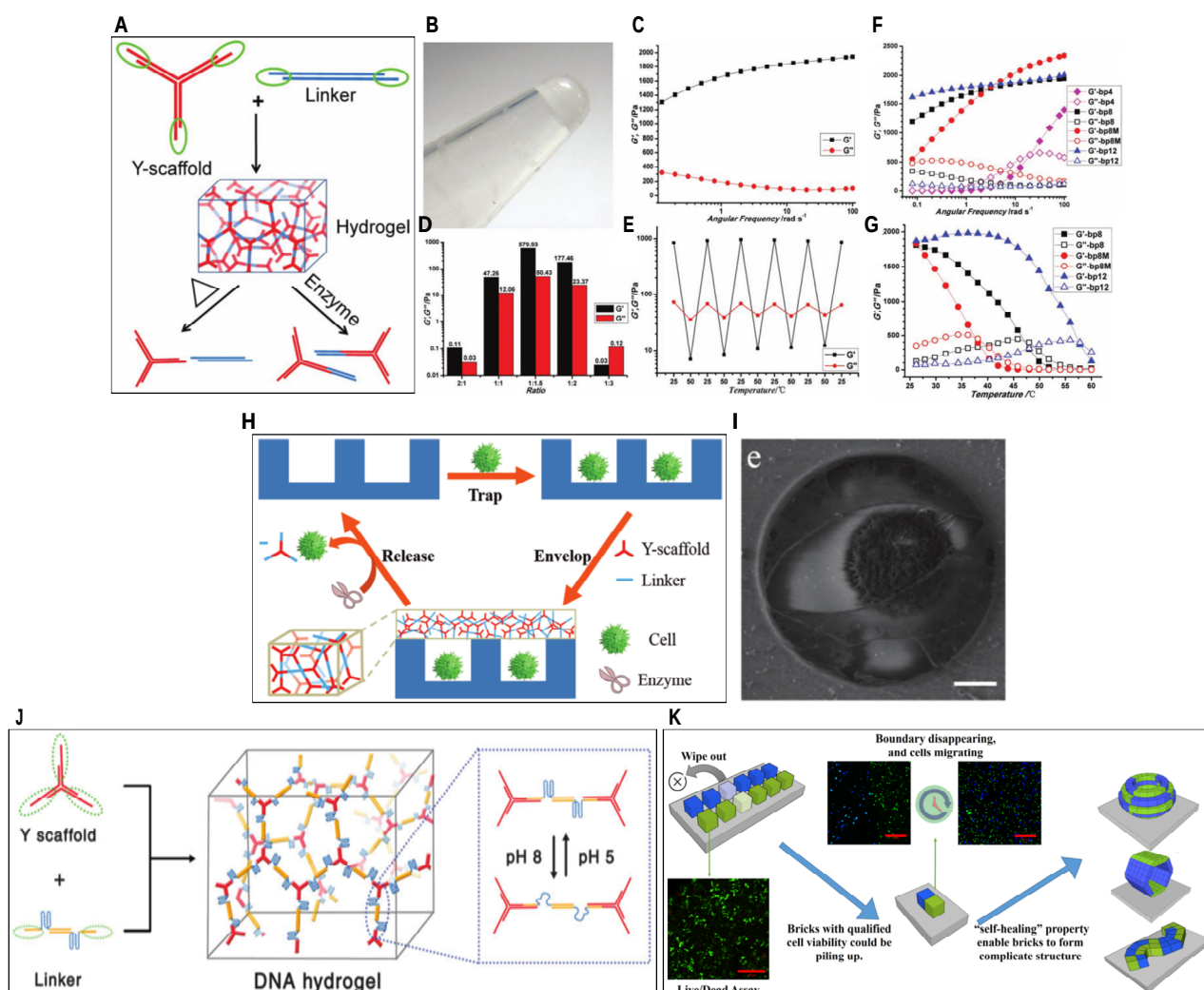


Figure 26 - DNA hydrogels stick-ends self-assembled aided by temperature. (A) DNA hydrogel formation using Y-DNA and a linear linker that crosslink by hybridization of their sticky ends (in green circles). The synthesized DNA hydrogels show thermal and enzymatic responsive properties¹²⁸. (B) DNA hydrogels prepared using 500 μ M of Y-DNA and 750 μ M of an 8 bp long sticky ends linker¹²⁸. (C) Frequency sweep test between 0.05 and 100 rad/s, strain of 1% at 25°C¹²⁸. (D) Rheological tests using 1 Hz frequency, 1% strain, 25°C for 3 min. Tests were done for DNA hydrogels with different molar ratios (Y-DNA/linker = 2:1, 1:1, 1:1.5, 1:2 and 1:3) with a total DNA content of 2% (w/v)¹²⁸. (E) Reversible thermal response of DNA hydrogels formed using 400 μ M Y-DNA and 600

μM of an 8 bp long sticky ends linker (1 Hz frequency, 1% strain, at 25°C and 50°C, for 5 cycles). Rheological properties of DNA hydrogels with different sticky ends (4, 8, 12 base pairs, with or without mismatch (M))¹²⁸. (F) Frequency sweep tests were carried out between 0.05 and 100 rad/s at 1% strain at 25°C¹²⁸. (G) Temperature-ramp rheological tests from 25°C to 60°C (2°C/min, 1 Hz frequency and 1% strain)¹²⁸. (H) Single cells trapped in PDMS microwells after DNA hydrogel formation. Specific restriction enzyme digests the DNA hydrogel and, as a result, cells are released on demand²⁵⁰. (I) SEM image of a single cell enveloped in the microwell covered with the DNA hydrogel cover (scale bar is 5 μm)²⁵⁰. (J) Insertion of a C-rich sequence in the linker (in blue). The conformational transition of the i-motif at different pH conditions had a great influence on the mechanical properties of the DNA hydrogel¹⁴³. (K) Brick-to-wall technology to fabricate 3D tissue-like structures. Different cell types are encapsulated in each DNA hydrogel brick and cultured for a period of time to eliminate damaged cells. Bricks are then combined to build the designed structures. No boundaries exist due to the DNA hydrogel self-healing properties (scale bar is 200 μm), thereby allowing for cell migration²⁵⁴. Adapted/reprinted with permission from ref. 128, Copyright © 2010 by John Wiley & Sons, Inc., ref. 143, Copyright © 2016 The Royal Society of Chemistry, ref. 250, Copyright © 2013 by John Wiley & Sons, Inc., and ref. 254, Copyright © 2017 American Chemical Society.

Other works exist in which this Y-DNA and linker strategy has been used to prepare physical pure DNA hydrogels, with fewer or more modifications. Applications involving the use of this type of hydrogel are various and include enzyme immobilization, bioelectrocatalysis without temperature increase to avoid enzyme denaturation²⁵¹, aptamer-induced targeting, and release due to the reduction of disulfide bonds in the cell cytosol's reducing environment for gene delivery applications²⁵². Other structures have also been applied in a similar manner. X-DNA for example was used to prepare a physical DNA hydrogel that through incorporation of G-quadruplex domains led to the development of a potential glucose/lactose detection device that mimics the peroxidase DNzyme catalytic activity in a hybrid cascade enzymatic reaction¹⁴². These X-DNA based physical hydrogels have been used for protein encapsulation/release.

Another approach to achieve the formation of a physical hydrogel is to endow ssDNA with several G-rich segments that, in the presence of cations, self-assembled into G-quadruplex domains. Using rolling circle amplification (RCA), a unidirectional replication process that results in multiple copies of circular nucleic acids, Huang *et al.*²⁵⁶ were able to cost-effectively produce large quantities of ssDNA with multi-repeated G-rich sequences that were then used to prepare a hydrogel crosslinked by intermolecular G-quadruplexes under physiological conditions (Figure 27). The coordination of the G-quadruplexes in the hydrogel with hemin resulted in a DNzyme with horseradish peroxidase (HRP)-like catalytic activity. Incorporation of glucose oxidase (GOx) into these G-quadruplex hydrogels was also found to be effective in detecting glucose in the hydrogel's surrounding environment.

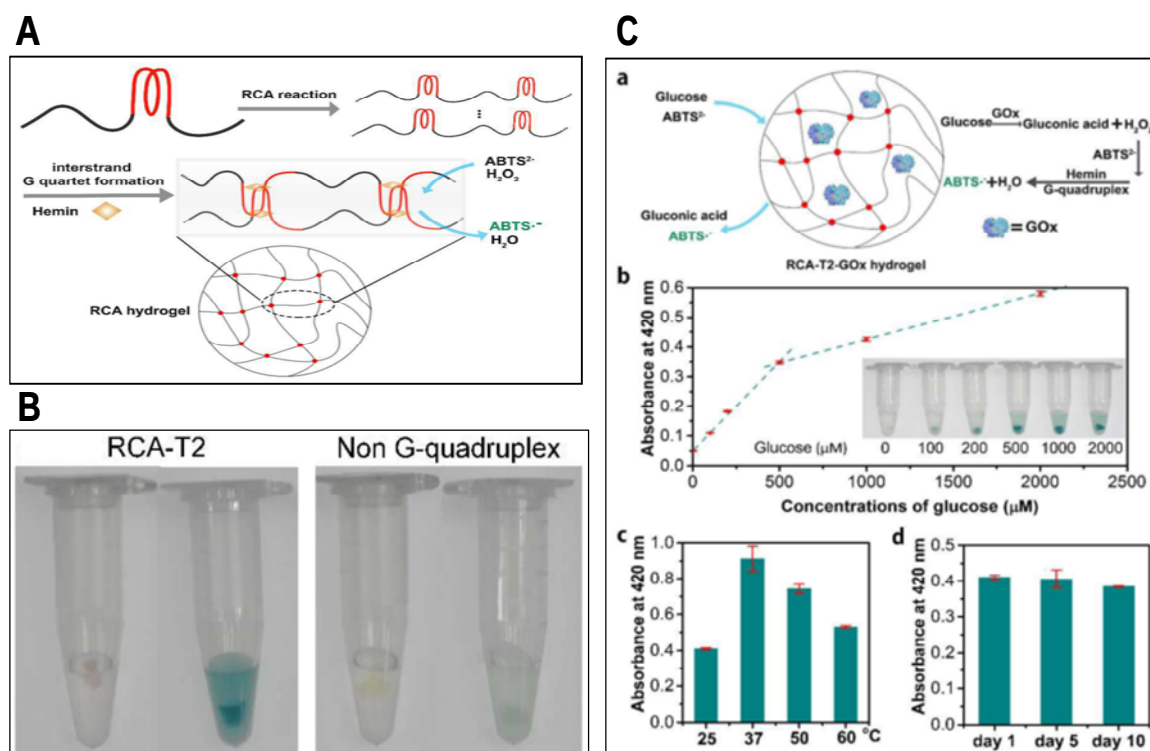


Figure 27 - G-quadruplex specific molecule recognition driven DNA hydrogel assembly. (A) RCA reaction to produce G-rich ssDNA, followed by interstrand G-quadruplex formation in the presence of hemin. The resultant DNA hydrogel displayed horseradish peroxidase (HRP)-like catalytic activity²⁵⁶. (B) The ABTS²⁻ colorimetric reaction before and after catalysis by the hydrogels²⁵⁶. (C) The sensitivity and stability of a RCA hydrogel incorporated with GOx (*i.e.* RCA-T2-GOx hydrogel): (a) working principle of the RCA-T2-GOx catalytic hydrogel; (b) quantitative and colorimetric detection of glucose by the RCA-T2-GOx hydrogel at different glucose concentrations; (c) quantitative detection of glucose by the hydrogel at different temperatures; and (d) quantitative detection of glucose by the hydrogel during storage²⁵⁶. Adapted/reprinted with permission from ref. 256, Copyright © 2017 The Royal Society of Chemistry.

In the same way that DNA hydrogels can be thermally produced and manipulated, certain DNA sequences can behave in response to environmental changes such as pH to form a hydrogel. As mentioned before, cytosine rich sequences have the ability to interact *via* hydrogen bonds in a C-CH⁺ mismatch with triple hydrogen bonds, forming what are known as i-motifs. Cheng *et al.*¹⁵¹ were able to efficiently prepare a hydrogel through the pH-triggered interlocking of i-motif domains (Figure 28). Several Y-DNA structures containing C-rich sticky ends were first prepared by temperature-induced hybridization. At high pH values, the Y-DNA structures suffered some electrostatic repulsion because the C-rich sequences were unprotonated and in a random coil conformation. Upon pH change to slightly acidic values, the Y-DNA structures instantly formed the hydrogel. Here, each Y-DNA was able to connect with up to three other Y-DNA structures in a ying-yang fashion. The gel-sol transition of the formed DNA hydrogel was verified upon drastic pH change, being confirmed by *in situ* encapsulated gold nanoparticles (AuNPs) that were released upon hydrogel dissolution. Hydrogel formation was found to be dependent on the pH and concentration of Y-DNA, where a pH less than 6

and a minimum of 0.5 mM Y-DNA was needed. This was confirmed by the hydrogel mechanical properties, where the shear-storage modulus (G') and shear-loss modulus (G'') presented comparable values ($G' > G''$ = solid, $G' < G''$ = liquid, $G' \approx G''$ = in-between state, hydrogel). As expected, the hydrogel was also thermosensitive, being stable only below its T_m . Due to the fact that the formation and stability of this hydrogel is pH dependent, it holds great potential application in both tumour and infection sites as both have acidic extracellular environments.

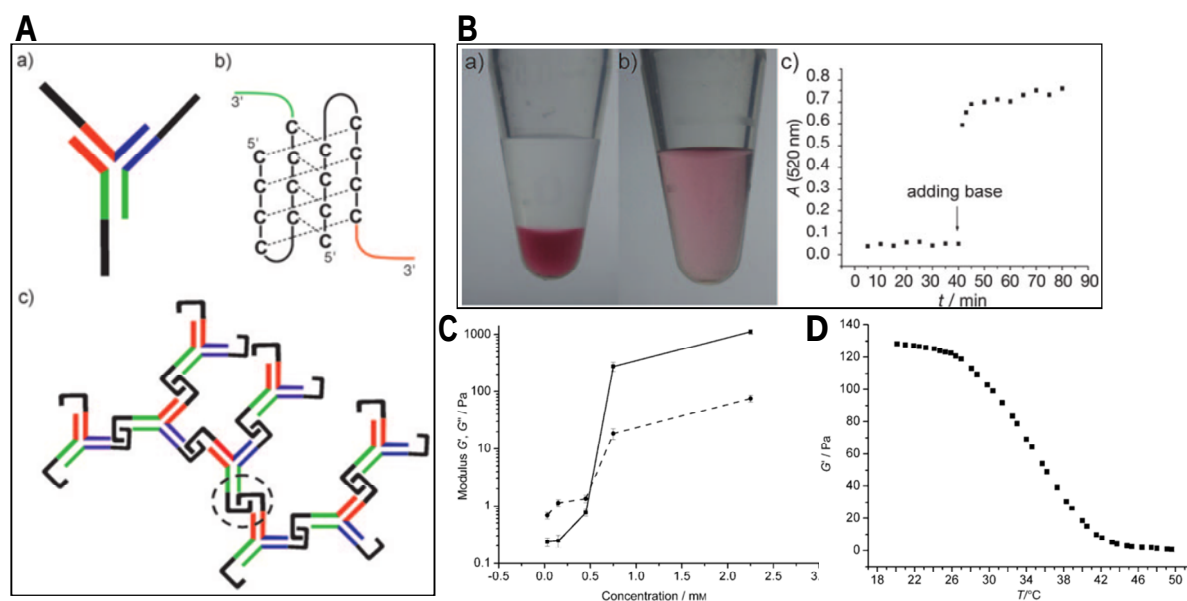


Figure 28 - i-motifs pH shift driven DNA hydrogel assembly. (A) Principle structure of the pH-responsive DNA gel, where (a) Y-DNA crosslinks through C-C mismatches forming (b) inter-Y-unit i-motifs; (c) DNA hydrogel made from the three-dimensional assembly of Y-DNA units. (B) Gel-sol transition switched by pH change. (a) 7 nm-sized AuNPs (pink) are trapped in the DNA hydrogel in a clear buffer solution (pH = 5). (b) After a change in the pH value of the buffer to pH 8.0, the AuNPs were released from the DNA hydrogel to form a uniform solution. (c) AuNP absorption at 520 nm of the upper part of the solution as a function of time, before and after the addition of NaOH. (C) Rheology tests of the DNA hydrogels prepared at pH 5.0, with storage modulus (G' ; solid line) and loss modulus (G'' ; dashed line) vs. the Y-DNA concentration (2.25, 0.75, 0.45, 0.15 and 0.03 mM) at 25°C. (D) G' value of the hydrogel as a function of temperature using a Y-DNA concentration of 0.60 mM.¹⁵¹ Adapted/reprinted with permission from ref. 151, Copyright © 2009 by John Wiley & Sons, Inc.

Small ssDNA fragments have the natural tendency to completely hybridize at room temperature in the presence of salts to stabilize the duplexes¹⁸. A very interesting work from Liao *et al.*¹⁴¹ combined DNA's ability to hybridize and the layer-by-layer technique (the LbL technique which will be later described in more detail in this review) taking advantage of the hybridization chain reaction (HCR) (Figure 29, A-B). HCR is based on the activation of a DNA hairpin by a ssDNA initiator. This activation leads to the exposure of a ssDNA sequence/region that will activate a second DNA hairpin, promoting a chain reaction. In this case, the oppositely charged polymers generally used in the LbL technique were substituted by complementary ssDNA strands that in turn were crosslinked

with a ssDNA sequence consisting of an ATP-binding aptamer. For the assembly of the DNA layers, tetramethylrhodamine dextran (TMR-D) loaded-calcium carbonate microparticles were first coated with positively charged poly(allylamine) (PAH). This served as a template for the addition of the ssDNA sequences, where the first interactions between the two components occurred through electrostatic interactions. Further addition of DNA hairpins in an alternate manner, always with washing steps in between to avoid assembly in solution, allowed for the build up of the DNA layers through hybridization of complementary segments *via* hydrogen bonding interactions. ATP-binding aptamer sequences were, this way, included in between the layers of the prepared DNA films. After the core of the formed DNA films was dissolved, the end result was a TMR-D loaded DNA microcapsule. Finally, TMR-D release from the microcapsule was mediated by ATP-aptamer association.

Another example of a hydrogel self-assembly construction based on HCR was presented by Wang *et al.*²²¹. The researchers constructed a tridimensional hydrogel with spacial and temporal control (Figure 29, C-D). In the so-called clamped-HCR (C-HCR) process developed here, three DNA strands were used, namely, two hairpin-containing structure (H1), a one hairpin structure (H2) and an initiator strand (I). Addition of the initiator, I, to a mixture of H1 and H2, resulted in its binding to the toehold region of H1 first, opening its long stem and short loop through displacement *via* a DNA displacement reaction (DDR). Activation of the H1 hairpin in this manner promoted the opening and subsequent activation of a H2 hairpin through the same mechanism, in turn promoting the opening of another H1 hairpin. Continuous cycling of this process, led to the final formation of the hydrogel.

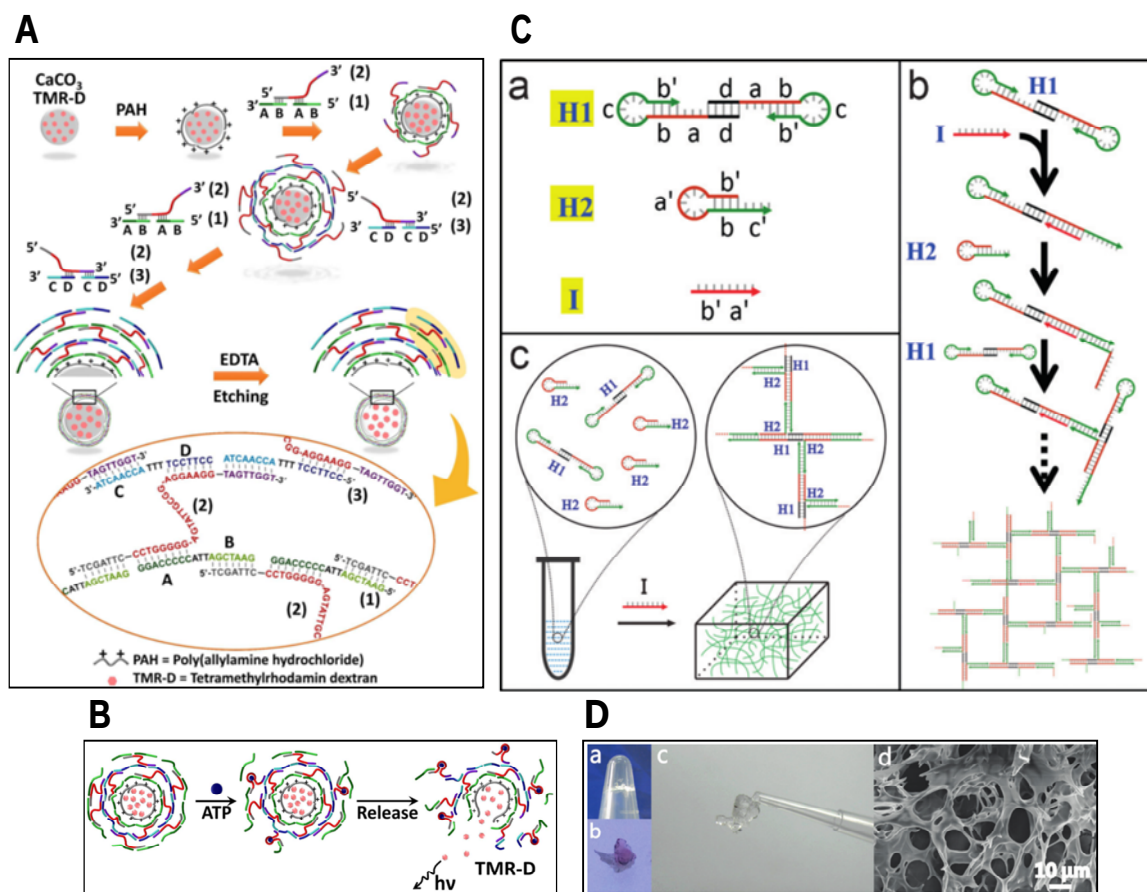


Figure 29 - DNA strands consecutive hybridization in a LbL-like fashion. (A) Preparation process of DNA microcapsules using CaCO₃ as a template and the subsequent LbL assembly of DNA¹⁴¹. (B) ATP-binding aptamer sequences labeled in red are embedded into the DNA films as stimuli-sensitive switches in the presence of ATP¹⁴¹. (C) Design principle of the DNA initiator triggered self-assembly process. (a) Structures of the H1-dimer and H2 hairpin strands, as well as the initiator strand I. (b) Details of the clamped-HCR (C-HCR) process initiated by I. (c) Representation of the gelation process²²¹. (D) Morphological characterizations and rheology tests of the DNA hydrogels, where (a) DNA hydrogel formed in a microtube. (b) 10 nm AuNPs trapped in DNA hydrogel, showing the typical red color. (c) Piece of DNA hydrogel stuck to a pipette tip. (d) An SEM image showing the inner structure of a dehydrated DNA hydrogel²²¹. Adapted/reprinted with permission from ref. 141, Copyright © 2015 American Chemical Society. and ref. 221 Copyright © 2017 by John Wiley & Sons, Inc.

Other physically crosslinked pure DNA-based hydrogels

Another means of producing pure DNA-based hydrogels physically crosslinked is based on enzymatic driven-polymerization process, where the synthesized DNA molecules are woven into condensed DNA forming a "birds nest" structure that demonstrates the typical features of a hydrogel. The ϕ 29 DNA polymerase can be used to build this type of materials since it has the ability to use a ssDNA molecule as a template to elongate primers or multi-primers, whether linear or circular. After elongation, the enzyme is able to displace the new DNA strand, amplify it to multiple copies and then weave these molecules into a condensed and entangled ball-like structure²⁵⁷. This

strategy was first applied in DNA hydrogel synthesis by Lee *et al.*¹³¹ in 2012. In the rolling circle amplification (RCA or R) process, a circular ssDNA molecule was hybridized with primer 1 to be elongated by ϕ 29 (Figure 30, A). The new elongated DNA (ssDNA 1) entered the multi-primed chain amplification (MCA or M) step by hybridization using two different primers (*i.e.* primer 2 and primer 3) to start the subsequent chain amplification process by ϕ 29, giving rise to ssDNA 2 (complementary to ssDNA 1) and ssDNA 3 (complementary to ssDNA 2). At the end, ssDNA 3 and ssDNA 1 have the same sequence. Thus, primer 2 is able to produce ssDNA 2 from ssDNA 1 and ssDNA 3, leading to chain amplification. The R and M processes could be extended for multiple times, being indicated as R_xM_y . The co-existence of so many ssDNA strands complementary to each other resulted in their hybridization, as well as non-covalent interactions and entanglements between them, with the end result being a hydrogel with unusual mechanical properties. This pure DNA hydrogel revealed itself to be a metamaterial, having unnatural and non-logical properties. This related specifically to the gel's solid and liquid-like properties in the presence and in the absence of water, respectively (Figure 30, B). The gel was shown to have shape-memory, by continuously returning to its original solid shape after undergoing complete deformation by the addition of water. In the absence of water and when exposed to air, the gel was observed to be deformed by surface tension and gravity. This effect was canceled when the gel was in water. Field-emission scanning electron microscopy (FESEM) of this meta-hydrogel unveiled a microstructure in the shape of a bird's nest, with internal nanopores (Figure 30, C). A very interesting application for this hydrogel would be the development of water switch devices. Here, the presence of water would solidify and shrink the hydrogel, disconnecting it from the electrodes and thus switching the electric circuit OFF (Figure 30, D). Switching ON the circuit would involve the inverse process, where the hydrogel has liquid-like properties. More recently, other authors have based their research on this type of hydrogel for use in applications such as protein synthesis²⁴³, hemo- and cytocompatibility for tissue engineering purposes²⁵⁸, as well as their use as biosensors for the detection of glucose through the incorporation of AuNPs with catalytic activity²⁵⁹.

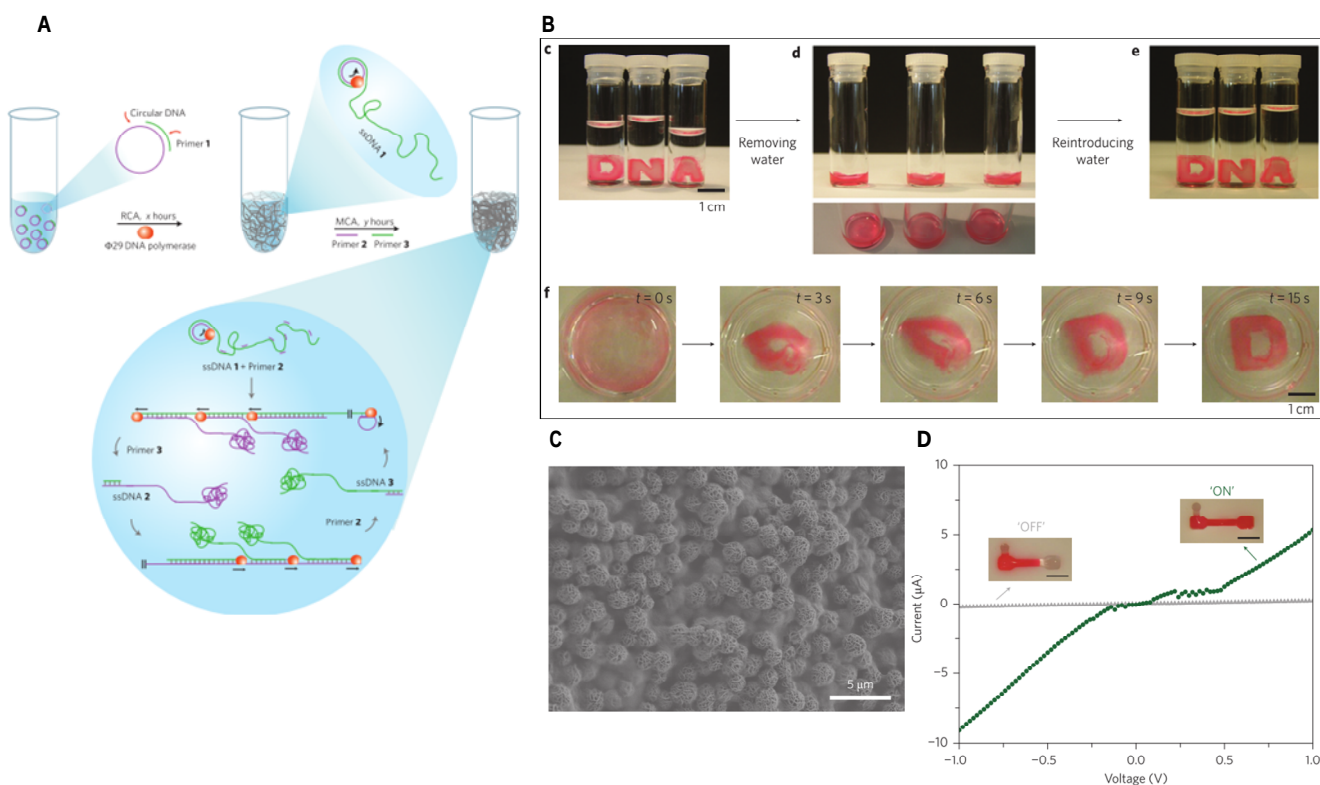


Figure 30 - RCA/MCA driven DNA hydrogel by elongation and weaving DNA molecules into densely packed bird nest structure with metamaterial properties. (A) DNA hydrogel synthesis by RCA and MCA. In the RCA process, a circular ssDNA template was first produced, followed by the addition of a complementary primer to produce elongated ssDNA products. Two other primers were then used in the MCA process for subsequent chain amplification. The long amplified products were then intertwined and woven to form a birds nest structure. (B) Liquid- and solid-like properties of the DNA meta-hydrogel, that is, when in water the hydrogel behaves like a solid and in the absence of water it flows like a fluid. (c) D- N- and A-shaped hydrogel and (d, e) its solid-like property was tested by removing and replacing water. (f) DNA hydrogel returning to its original shape after reintroducing water at 25°C as a function of time. (C) SEM images of the R_8M_{16} DNA hydrogel. (D) Electric circuit switch formed using the liquid- and solid-like properties of the DNA meta-hydrogel. When the DNA hydrogel (containing 10 nm AuNPs) has liquid-like properties, the circuit can be covered by the gel (green circles, ON). In the presence of water, the gel returns to its original shorter shape, resulting in the gel moving away from the electrode within seconds (grey triangles, OFF). Scale bar is 5 mm (insets).¹³¹ Adapted/reprinted with permission from ref. 131, Copyright © 2012 Springer Nature.

Lee *et al.* developed another type of pure DNA-based hydrogel made up of entangled fibres¹⁴⁸. DNA is a flexible linear polymer that through non-covalent interactions can form condensed structures such as interwound supercoils, bundles, clusters of toroids and multiple loops and knots (Figure 31). Promoting this condensation process are the ionic strength and the DNA concentration in solution. Ionic liquids (IL), which correspond to salts that can be found in a liquid state at very low temperatures or even at room temperature due to the weak coordination between ions²⁶⁰, were thus used by Lee and coworkers in their work¹⁴⁸. In fact, room temperature ionic liquids (RTILs) were found to produce the most suitable conditions to promote DNA fibre coagulation in wet spinning by condensing, intertwining and entangling the fibres in a random and disordered manner. This resulted

in the formation of a hydrogel made up of DNA fibres. After washing, it was confirmed that no salts were left in the hydrogel and a pure DNA hydrogel, without loss of B-conformation or denaturation was thus obtained. The hydrogel was observed to swell normally in water due to hydrogen bonds between the DNA phosphate groups and the water molecules. In the presence of cations, the hydrogel contracted due to charge neutralization of the phosphate groups of the DNA strands resulting in a decrease in the electrostatic repulsion between them. In addition²⁶¹, at lower pH values, hydrogel contraction was greater because hydrolysis and DNA base oxidation led to denaturation. When using higher pH values, hydrogel denaturation followed by fragmentation and then dissolution was also reported to occur. The tightly condensed DNA fibres in the hydrogel made it nearly impossible for restriction endonucleases (DNases) to bind to and cleave DNA. In this way, the DNA fibre hydrogel was found to be resistant to degradation.

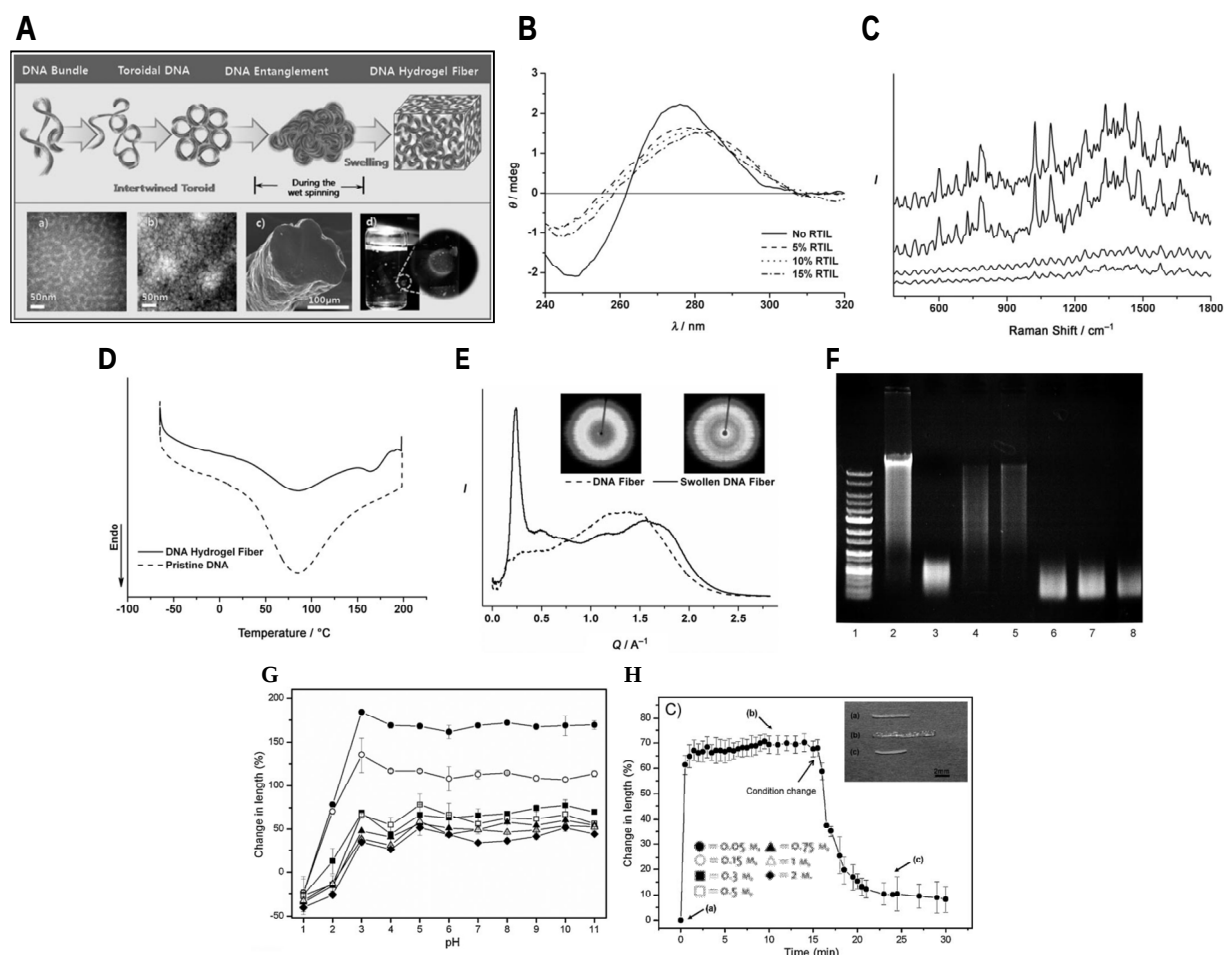


Figure 31 - DNA dense compaction promoted by RTIL into a hydrogel. (A) Preparation of the DNA hydrogel fibre, with (a) DNA condensation in torus-shaped and rod-like morphology, (b) toroids circumferentially wrapped around by the RTIL, (c) morphology of the DNA hydrogel fibre and (d) characterization of the swollen DNA hydrogel¹⁴⁸. (B) Circular dichroism of DNA (0.1 mg/mL) at different concentrations of the RTIL shows that DNA did not loose the B-conformation¹⁴⁸. (C) Polarized (parallel, perpendicular, and oblique to the axes) Raman spectra (514.5 nm excitation) for the DNA fibre (intensities correspond to the relative orientation of the transition moment and to rotary polarization)¹⁴⁸. (D) Thermal stability of DNA fibres by differential scanning

calorimetry (DSC)¹⁴⁸. (E) X-ray scattering profiles of the DNA hydrogel fibre in a dry and a swollen state¹⁴⁸. (F) Gel electrophoresis of the effects of restriction endonuclease (DNase) on DNA alone and on the DNA hydrogel fibre (lane 1: marker; lanes 2 and 3: DNA and spinning solution, respectively, without DNase; lanes 4 and 5: DNA digested with XbaI and XhoI, respectively; lane 6: DNA hydrogel fibre without DNase; lanes 7 and 8: DNA hydrogel fibre digested with XbaI and XhoI, respectively)¹⁴⁸. (G) The change in length of DNA hydrogel fibres in solutions with different pH and $[Na^+]$ ²⁶¹. (H) Kinetics of the change in length of DNA hydrogel fibres on switching $[Na^+]$ from 0.15 to 1 M at a pH of 7: (a) dry DNA hydrogel fibres, (b) DNA hydrogel fibres in solutions at a pH of 7 and $[Na^+] = 0.15$ M, and (c) DNA hydrogel fibres in solutions at a pH of 7 and $[Na^+] = 1$ M²⁶¹. Adapted/reprinted with permission from ref. 148, Copyright © 2008 by John Wiley & Sons, Inc. and ref. 261, Copyright © 2009 by John Wiley & Sons, Inc.

2.3. Volume and phase transitions of DNA-based hydrogels upon external stimuli

DNA-based hydrogels can respond to physical (e.g. temperature, radiation, mechanical), chemical (e.g. pH, metallic ions, organic reagents) or biological (e.g. biomolecules such as glucose, enzymes, thrombin, nucleic acids, ATP) stimuli¹¹⁷. In fact, almost every DNA-based hydrogel previously referenced has a macroscopic level response to external environmental stimuli, whether it is physical (e.g. change of volume/phase or change of color/fluorescence) or chemical/biochemical (e.g. when a DNA hydrogel is combined with recognition and/or catalytic entities). This ability to respond to an input in a more general or in a specific manner makes DNA-based hydrogels "smart" or "stimuli-responsive" soft materials with tunable properties, thus being of great versatility in terms of applications.

In this section, due to their relevance, hydrogel volume/phase transitions triggered by external stimuli will be discussed. More specifically, the hydrogel response can correspond to (i) a volume transition where the size, porosity and mechanical properties change without the disintegration of the gel, or (ii) a phase transition, usually from the gel to the liquid state (gel-sol transition).

Usually, volume transitions (Figure 32, A) do not constitute the destruction of a hydrogel since the volume occupied by the gel will only increase (*i.e.* swell) or decrease (*i.e.* shrink). Here, structural elements such as chemical crosslinking points through covalent bonds, physical crosslinking through non-covalent bonds or the combination of both, all play a role in ensuring the maintenance of the hydrogel structure. In the case of hydrogels displaying chemical and physical crosslinking combined, cooperative forces will play a role in maintaining the different entities in the structure. A volume transition can be achieved by momentarily disrupting one of the entities, while the other entity is maintained in order to preserve the tridimensional structure and shape-memory of the hydrogel. On the other hand, phase transitions (Figure 32, B) involve reversible or irreversible gel-sol transitions where the tridimensional nature of the hydrogel is lost either momentarily or irreversibly. Note that the phase transitions depend on the type and intensity of the forces that are

established among the components of the hydrogel and are influenced by the environment to which it is exposed²⁶².

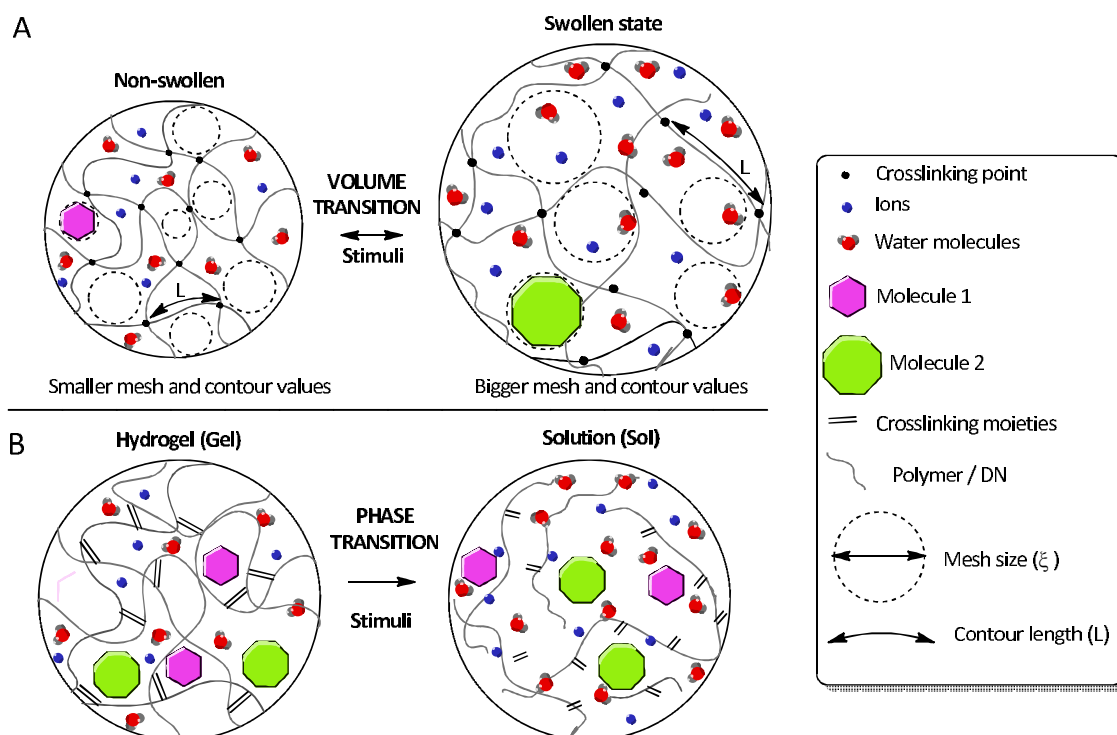


Figure 32 - Volume and phase transitions of hydrogels, upon specific stimuli. (A) Volume transitions: reversible from non-swollen to swollen state, upon exposure to the right stimuli. In the non-swollen state, the mesh and size is smaller. In the swollen state, on the other hand, the mesh size increases leading to the release of the molecules. (B) Phase transitions: usually irreversible between gel and solution state, where the loaded molecules are released under specific stimuli.

Volume transitions

Hydrogels have a universally intrinsic property in which they swell/shrink when exposed to solutions having different concentrations and/or composition from the one in which they were prepared^{25,88,121–123,131,146–148,153,155,158–160,162–164,166,167,169,171–174,188,209,215,235,261}. This could be observed in most of the previously referenced works where the behaviour of different DNA-based hydrogels was evaluated in the presence of water^{131,209}, ionic aqueous solutions such as buffers¹⁷¹, organic/water mixtures^{122,162}, surfactants or polyelectrolytes¹⁷³. In all cases, the volume transitions were found to be dependent on ionic strength and/or the presence of solutes in solution, mostly influencing the osmotic pressure, electrostatic interactions between oppositely charged groups, repulsion/attraction of the ionized groups or the hydrophilic/hydrophobic interaction between the outside species and the network components of the hydrogel in a passive manner. Indeed, hydrogels prepared with polyelectrolytes revealed that gel swelling was more affected by osmotic pressure and electrostatic

repulsion of the backbone²¹⁵. In contrast, hydrogels prepared with non-charged polymers were unaffected by pH or ionic strength as no interactions with the neutral polymer occurred²⁶³.

Such swelling/deswelling behaviour has allowed researchers to evaluate the mechanical properties of hydrogels in terms of mesh/pore size, strength, viscoelasticity, deformation, among others, using swelling, rheological and mechanical tests¹⁶². However, the extent of volume transition is not only dependent on the environmental conditions but also on the network's chemical nature and degree of freedom. Importantly, the degree to which a gel will swell/shrink is proportional to the crosslinking density, that is, the distance between the crossing points (contour length, L)¹⁷³.

Hydrogels can be particularly designed to suffer specific volume transitions under specific external stimuli (can be activated regarding volume transitions). For example, a general way to achieve a volume transition is to endow the DNA hydrogel with protonable functional groups such that, upon pH change, these will be (de)protonated and will promote either attraction/repulsion interactions between the chemical groups that are present^{148,179,222,261}. The introduction of redox-responsive groups such as disulfide bonds¹³⁷, thermo-responsive polymers such as PNIPAM^{133,202,203} or light-responsive molecules, such as azo-derivatives or EGDE^{158,185}, will also influence the swelling/shrinking response of the gel to the specific triggers. Due to the unique properties of the DNA molecule and to the richness of structures that it can form (e.g. ssDNA, toeholds, aptamers, i-motifs, G-quadruplexes, triplexes and DNAzymes), DNA's presence in a hydrogel allows the use of even more sophisticated methods to actively promote and tune changes in volume. Some representative examples of strategies that can be used at this level are given below.

The existence of DNA sequences able to hybridize with a duplex *via* Hoogsteen base interactions²⁶⁴ to form a triplex (Figure 33, A), permitted researchers to obtain duplex/pH-responsive triplex DNA hydrogels^{189,192}. These hydrogels were formed under specific pH conditions through cooperative forces between the duplex and triplex structures. Upon an environmental change of the pH, the triplexes were disrupted. The duplexes on the other hand, maintained the hydrogel properties (on the condition that they were never disrupted) and the shape-memory capacity. Moreover, when under the right pH conditions, the formation of the triplex back into the same shape and in the same manner was once again possible. Thus, the DNA hydrogel developed here was able to switch between a defined shape and a more shapeless, quasi-liquid state in a pH-dependent manner.

Other pH-switchable structures that may be incorporated into DNA hydrogels are i-motifs. These C-rich DNA sequences can undergo a mismatch hybridization process that is promoted by pH protonation^{143,144,207} or by silver ions that have been combined with a chelating cysteamine counter trigger²²⁰. As previously mentioned, the cooperation between duplexes and i-motifs has led to the production of pH-reversible shape-memory hydrogels (Figure 33, B).

The guanosine-rich G-quadruplexes are also stimuli-responsive structures. They respond to the presence of different ions, being very selective towards potassium, lead or strontium²²⁰. In contrast to i-motif containing hydrogels, reversibility in G-quadruplex-based hydrogels is only possible upon the cyclic addition of a counter-trigger such as DOTA or KP that has the ability to chelate ions (Figure 33, C). The combination of G-quadruplexes and i-motifs has been shown to be a multi-trigger stimuli-responsive approach in the DNA hydrogel field, in which the hydrogel produced is able to respond to several different stimuli types^{191,203,220}. Furthermore, when combined with hemin, an iron-coordinated porphyrin, the G-quadruplexes acquire catalytic activity, converting a specific substrate to a detectable reaction product.

Volume transitions have also been reported in DNA hydrogels that have ssDNA grafted onto a polymeric network. Here, the transitions occur as a result of the hybridization of ssDNA with a complementary strand (Figure 33, D). More specifically, the grafted ssDNA can be completely available for hybridization to occur or it can exist as a stem-loop (hairpin loop). Irrespective of the DNA form used for hybridization, several DNA hydrogels of this type have been evaluated in terms of their different mechanical responses (e.g. swellability and rigidity), as well as their ability to detect specific complementary ssDNA, thus making them DNA-responsive hydrogels^{137,183,191,195,197,198,208}. It was found that the swelling/shrinking behaviour of the hydrogels was affected by the lower interaction of dsDNA relative to ssDNA with water molecules¹⁹⁷, by the higher rigidity in dsDNA than in ssDNA^{195,208}, by change in the contour length by strand displacement^{137,191,195,208} and dsDNA length stability dependency¹⁸³.

gel-sol transition without maintaining any shape-memory properties. The hydrogel building blocks will end up dissolved in the surrounding liquid medium.

As described before, most of the times, the network in DNA-based hydrogels involves the establishment of physical interactions among its components, namely hydrogen bonds associated to base pair recognition. As such, parameters that interfere with these physical forces may promote a gel-sol transition. This can be done directly by increasing the temperature which will cause DNA denaturation^{25,123,128,145,149,154,157,162,169,222,223,235,253,265}. This process can be reversed by decreasing the temperature to values lower than T_m or to room temperature.

At room or physiological temperature, phase transitions can be actively promoted in DNA-based hydrogels by the inclusion of specific chemical moieties within their structure that will specifically respond to external stimuli. These stimuli-responsive DNA-based hydrogels can respond to physical or chemical inputs including light, pH, light, or differentiations/molecules (e.g. ssDNA, ions or small drugs) that will interact with specific "receptors" in the gel. For example, as previously described, the inclusion of photo-sensitive moieties in the hydrogels, like EGDE^{88,158,161,165,168} and azo-derivatives¹⁸² makes them responsive to light. Kang and coworkers reported a new type of hybrid hydrogel crosslinked by DNA with reversible photo-controllable phase transition. This material was functionalised by the incorporation of azobenzene moieties into the DNA crosslinker. Upon irradiation, the azobenzene isomerized (trans/cis isomerization) which resulted in the impossibility of hybridization between the azobenzene-functionalised DNA crosslinker and the complementary DNA strands. On the other hand, DNA-based hydrogels possessing either i-motifs made up of C-rich DNA sequences^{130,133,151} or triplexes involving Hoogsteen base interactions¹³⁹ (Figure 34, A-B) will display pH-sensitive properties, being able to switch ON and OFF between gelation and dissolution through simple pH changes.

At room or physiological temperature, DNA-based hydrogels may also suffer phase transitions (reversible or permanent) mediated by specific molecules. The recognition of specific ssDNA strands by DNA hydrogels (Figure 34, C), for instance, can also lead to gel dissolution when the essential component for gelation is a ssDNA linker^{145,150,186–188,210,212,214}. Here, the presence of a toehold in the ssDNA linker will facilitate dissociation. The dissociation starts at the toehold, followed by branch migration, a random walk of the junction between the linker and the displacement strand, until the toehold strand is displaced²⁶⁶. Another simple approach to produce DNA-based hydrogels capable of phase transition is to include G-rich DNA sequences that are able to self-assemble in the presence of certain ions. In the work of Lu *et al.* (Figure 34, D), G-quadruplexes were used in the crosslinking process which was stabilized by the potassium ion. The addition of a K^+ chelator (the counter-trigger) induced the dissolution of the hydrogel^{132,191}. The same happens with Ag^+ -stabilized

C-C mismatch DNA hydrogels (Figure 34, E), where the addition of cysteamine as the counter-trigger results in hydrogel dissolution^{133,138}.

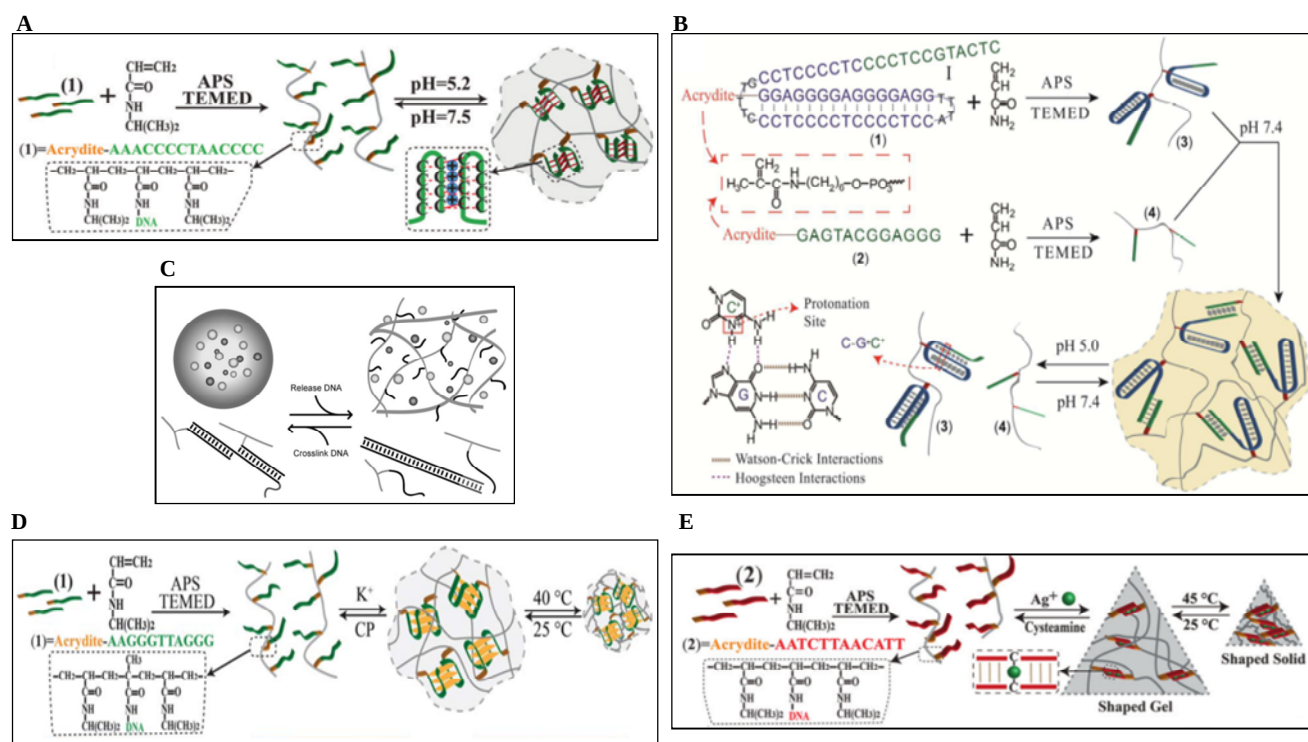


Figure 34 - Switchable DNA hydrogel phase transitions promoted by i-motifs, triplexes, G-quadruplexes and duplexes. (A) DNA-modified pNIPAM copolymer, where the i-motif-crosslinked hydrogel undergoes pH-switchable formation and dissociation¹³³. (B) Formation of DNA hydrogel triggered by pH change, where crosslinking of the polymer chains occurs as a result of duplex Watson-Crick interactions and Hoogsteen triplex interactions¹³⁹. (C) DNA-crosslinked polyacrylamide hydrogel dissolved by a DNA release strand. The inverse process involves the formation of a DNA "waste" duplex composed of crosslinking DNA strands and the release strand. The gel can be reorganized by adding the crosslinking strand again²¹⁰. (D) Thermosensitive G-quadruplex-crosslinked acrydite/pNIPAM copolymer switchable hydrogel, which undergoes reversible and cyclic transitions across solution-hydrogel-solid states²⁰³. (E) Thermosensitive ssDNA-grafted pNIPAM copolymer with Ag⁺-assisted crosslinking of the ssDNA strands to obtain a hydrogel. Separation and reformation is possible upon treatment with cysteamine and Ag⁺ ions, respectively¹³³. Adapted/reprinted with permission from ref. 133, Copyright © 2014 by John Wiley & Sons, Inc., ref. 139 and ref. 203 Copyright © 2015 The Royal Society of Chemistry, and ref. 210, Copyright © 2007 by John Wiley & Sons, Inc.

In the case of the aptamer-based DNA hydrogels, gel dissolution occurs after aptamer-target association^{127,141,144,190,211,213,217}. The process here involves the DNA linker approach, where two non-complementary ssDNA grafted polymers are crosslinked by a complementary ssDNA (Figure 35, A-C). The DNA linker contains the specific aptamer sequence^{57,227} that exhibits an antibody-like behaviour towards a specific target molecule, the "antigen". In the presence of the antigen trigger and following binding, the aptamer will undergo a conformational change. This will lead to disruption of the hydrogen bond established between the aptamer linker and the complementary grafted ssDNA, and the subsequent dissolution of the gel. This strategy has been applied for the detection of small

molecules such as ATP^{141,144,211,213} or cocaine^{127,144,217}, as well as ions (Mg^{2+} , Zn^{2+} , Cu^{2+})¹⁹⁰. In both cases, the target molecules/ions are detectable because their binding to the aptamer results first in hydrogel disruption and then content release (colorimetric detection).

The disruption of covalent bonds within a gel induced by an external signal may also lead to its dissolution in the surrounding medium. Although not regarded as a "pure" phase transition since covalent bonds are involved in the process, a gel-sol transition may however be observed. This is the case of hydrogels that contain a DNAzyme as a component in their structure^{129,193,218}. The approach here also consists of a ssDNA strand grafted onto a polymer backbone that hybridizes either to its complementary ssDNA or to a ssDNA linker with a recognition-like property (usually towards ions) and an additional catalytic site that confers it enzyme-like activity (Figure 35, D-F). In the presence of ions such as Cu^{2+} , Pb^{2+} and Ln^{3+} , the DNAzyme will recognize the ion(s). The catalytic site of the enzyme will thus be activated, leading to the cleavage of either the ssDNA or the DNA linker to which it is hybridized. This will finally result in the dissolution of the DNA hydrogel.

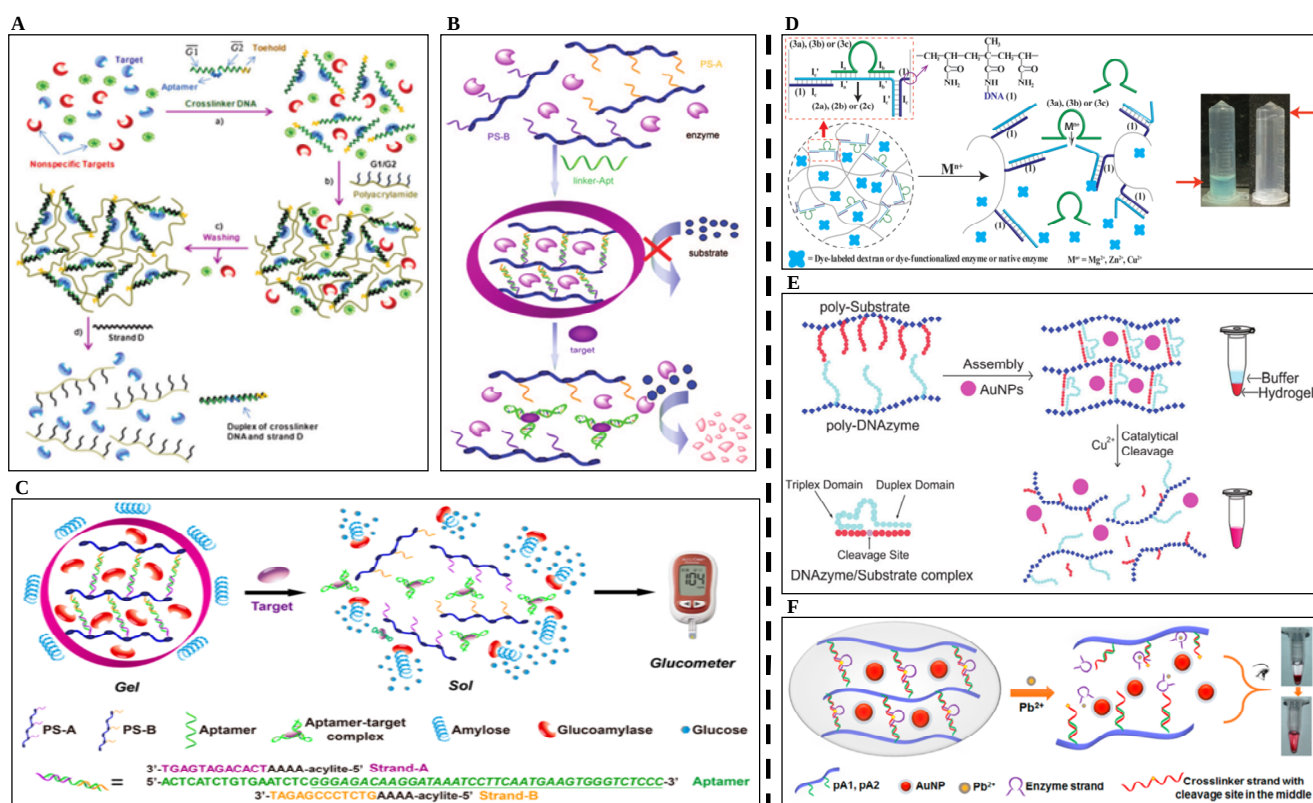


Figure 35 - Controlled release of DNA hydrogel encapsulated catalytic molecules promoted by targeted recognition of specific molecules. (A-C) Aptamer-based DNA hydrogel for specific target recognition. (A) ATP, where successful aptamer-ATP binding is visualized by the dissolution of the hydrogel²¹³. (B) Cocaine, where the cocaine-aptamer leads to the dissolution of the hydrogel, releasing its amylase load. The amylase then breaks amylose down into a sugar resulting in a color change from yellow to dark blue¹²⁷. (C) Same principle as the cocaine detection method but used to detect non-glucose targets through the detection of glucose²¹⁷. Applied to portable devices with added advantages (e.g. rapid, on-site, user-friendly and cost-effective). (D-F) DNAzyme-based DNA hydrogel for specific metal ion detection. (D) Magnesium, zinc and copper ions, where ion binding to the metal ion-dependent DNAzyme strands results in cleavage of the substrate and release of

the entrapped dye-labeled dextran or with enzymes. Photograph of the dissolved hydrogel (left: blue gel at the bottom) and the crosslinked hydrogel (right: clear on top)¹⁹⁰. (E) Copper ion responsive DNAzyme crosslinked hydrogel. In the presence of Cu²⁺, the DNAzyme cleaves the crosslinking substrate strand and releases the encapsulated AuNPs into the supernatant allowing for their visual detection¹²⁹. (F) DNAzyme crosslinked hydrogel for visual detection of lead ions, following the same principle as before²¹⁸. Adapted/reprinted with permission from ref. 127, Copyright © 2010 by John Wiley & Sons, Inc., ref. 129, Copyright © 2011 The Royal Society of Chemistry, ref. 190, Copyright © 2015 American Chemical Society, ref. 213, Copyright © 2010 The Royal Society of Chemistry, ref. 217, Copyright © 2013 American Chemical Society and ref. 218, Copyright © 2014 American Chemical Society.

Another example of a gel-sol transition involving the break of covalent bonds is when disulfide bonds are introduced within DNA-based hydrogels that make them sensitive to redox environments. Nanogels for gene therapy applications made up of DNA have also been shown to benefit from having disulfide bonds incorporated into their structure, where their content was only released after exposure to the intracellular reducing agent, glutathione (GSH)²⁵².

2.4. Applications of DNA-based hydrogels

DNA-based hydrogels may be used in different fields ranging from environmental sciences to biomedicine (Figure 36), not only taking advantage of the characteristics of the DNA molecule itself, but also of their tridimensional network structure and stimuli responsiveness properties. Although DNA-based hydrogels' applications have been evidenced throughout the previous section, they will be here summarized for a better overview of the potential associated with these "soft" and "smart" materials.

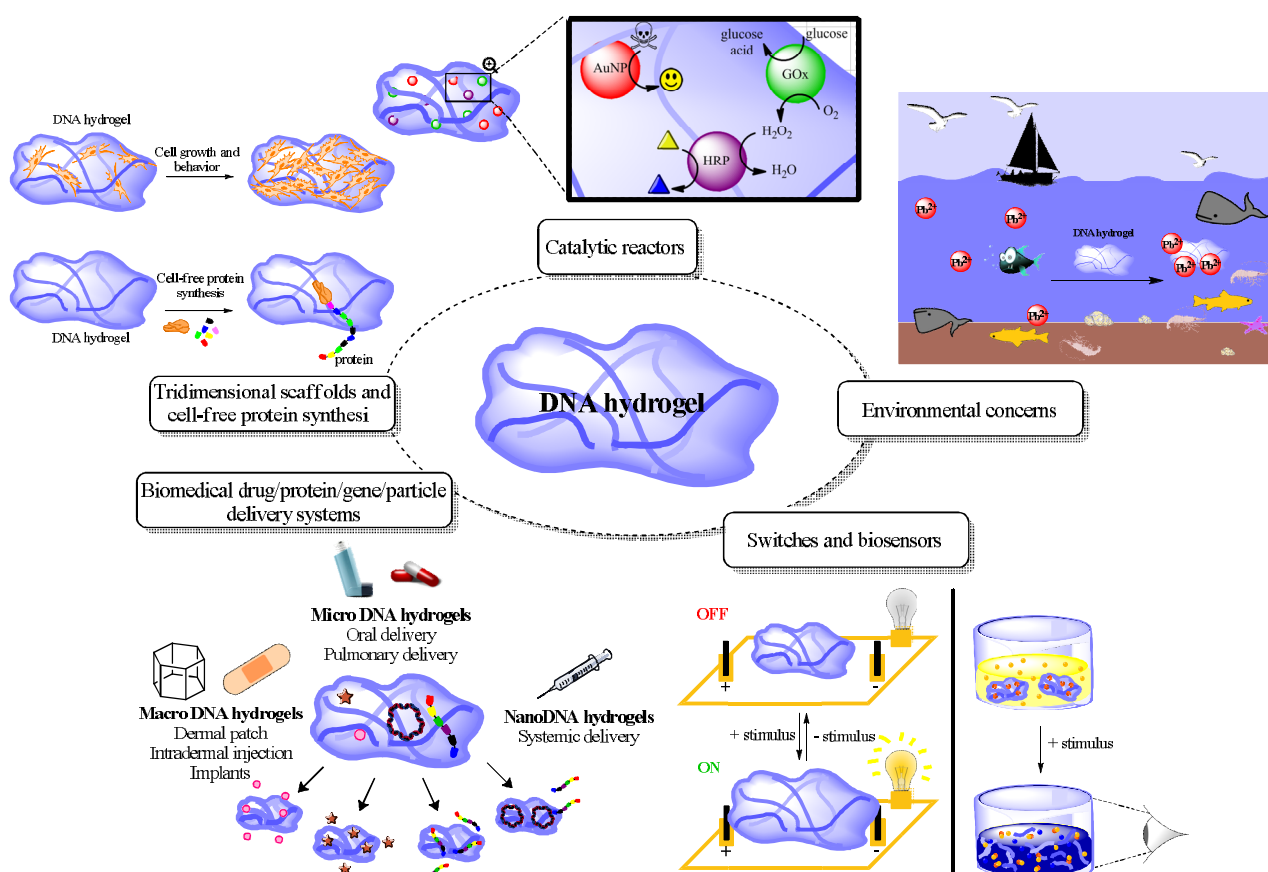


Figure 36 - Main applications of DNA hydrogels, from environment to biomedical. As catalytic reactors, it is implicit that DNA hydrogels encase particles (e.g. nanoparticles or enzymes) with catalytic properties that will convert a toxic substrate into a less harmful molecule or a biomolecule into a detectable product. The presence of mutagenic molecules (e.g. EtBr) or heavy metal ions (e.g. Pb^{2+}) in the environment will trigger a counterattack reaction when using DNA hydrogel strategies, as the affinity of these molecules/ions for DNA will be exploited. The stimuli-responsive behaviour of DNA hydrogels towards a series of different stimuli has opened the doors to the manipulation of hydrogel shrinkage/swelling degree and its use as ON/OFF switches and as biosensors that upon the dissolution and release of enzymes will convert its substrate into a colored product allowing for visual detection. The use of DNA hydrogels as a delivery system or platform for the delivery of nanoparticles (pink circles), drugs (brown stars), proteins (colored chains) or nucleic acids (represented by a pDNA). Also, the size of the DNA hydrogel will determine its administration route. A very promising application of DNA hydrogels is its use as a tridimensional scaffold for cell culture to study cell behaviour and intercellular communication, as well as its use as a cell-free protein synthesis platform where genes can be incorporated into the DNA hydrogel that when in the presence of the appropriate protein synthesis machinery will give rise to proteins.

DNA-based hydrogels in environmental applications

An important use of DNA-based hydrogels is the removal of pollutants from the environment. Indeed, small molecules such as EtBr, acridine orange (AO) and Hoechst33258 (H33258), among many others, are known to be able to intercalate between the DNA bp or to bind the minor/major grooves of DNA³⁸. Thus, they are considered mutagens that may present some catastrophic carcinogenic effects. In fact, the production and usage of such small problematic

molecules in industry, academic organizations, research institutions and waste treatment facilities may result in some form of loss of these pollutants to the environment.

Another application of these hydrogels in the environmental field is related with their use in the detection of heavy metals like Hg^{2+} . Regarding this, an example deserves to be mentioned, namely an approach based on the different color fluorescent properties of SYBR Green I in the presence of ss or dsDNA^{126,215}. Briefly, due to the affinity of DNA's thymidine base towards Hg^{2+} , the T-rich sequences grafted into the DNA hydrogel suffer a transition from linear to hairpin (T-T mismatch) in the presence of these ions. The addition of the SYBR Green I dye to the Hg^{2+} -containing DNA hydrogel displays a green fluorescence signal. This is in contrast to DNA hydrogels in the absence of Hg^{2+} , where the signal displayed is yellow. After Hg^{2+} detection has been confirmed, the immobilized ions can be removed from the DNA hydrogel thus allowing for its regeneration for further Hg^{2+} removal.

DNA-based hydrogels as catalytic reactors

The DNA hydrogel tridimensional network allows for the entrapment and permanent immobilization of molecules, leading to the potential creation of catalytic device. Indeed, the DNA hydrogel network has been used for AuNP size-controllable synthesis^{164,166,167} for posterior catalytic applications including Ag^+ reduction for gel staining²⁰⁰, and conversion of p-nitrophenol in polluted industrial residual waters to aminophenol^{166,167}. Also, the production of DNA hydrogels at room temperature is an attractive feature for the *in situ* encapsulation of biomolecules such as enzymes^{251,256,259}. This is because biomolecule inactivation by heat or denaturing agents is avoided, making the hydrogel an ideal reactor for enzymatic reactions.

Another example involves the incorporation of G-quadruplexes^{132,203} and DNAzymes^{129,142,190,193,218,219} into the DNA hydrogel structure, which confers catalytic activity to the gel while it is being formed. In this manner, the catalytic entity forms part of the hydrogel itself and is not imbibed in it.

DNA-based hydrogels as biosensors and switches

DNA-based hydrogels have been used to detect ssDNA sequences in a wide range of biomedical applications. This strategy has been used to create devices such as microarray chips^{53,267}. It has also been used in DNA sensing devices to detect specific ssDNA sequences with extreme sensitivity allowing for the distinction of mismatches to discriminate between normal and mutant alleles and for diagnostic purposes²⁰¹. The process involved in these techniques is focused on ssDNA

hybridization, which is very specific and goes down to the recognition of base mismatches. Generally, the recognition/hybridization events associated with these techniques can be detected by fluorescence^{194,201}, electrochemical measurements^{140,202,242}, swelling behaviour^{197,198} and AuNP and silver staining²⁰⁰.

In addition to biotechnological and biomedical applications¹⁹⁷, ssDNA detection may just be a small step or trigger (e.g. DNA-triggered devices promoted by ssDNA, toehold or aptamer hybridization) to something bigger (e.g. drug, gene or particle sustained delivery). The use of the aptamer concept and its ability to recognize a specific target, forming an aptamer-target complex, may be used as an innovative method for the separation and purification of the target itself²¹³.

DNA-based hydrogels can also be applied as switch devices. In a work previously mentioned, a DNA hydrogel was shown to respond very quickly to a water-based trigger. More specifically, the gel was observed to behave as a solid and as a liquid in the presence and in the absence of water, respectively¹³¹. This change between the liquid-solid states played a role in connecting (ON; without water) and disconnecting (OFF; with water) the electrodes in an electric circuit, allowing the current to flow or not accordingly.

DNA-based hydrogels as delivery systems

In the same way that molecules can be immobilized in the DNA hydrogel structure turning it into a reactor, the same strategy can be followed to load the hydrogel with molecules for later release in a targeted, controlled and sustained manner. Currently, most DNA hydrogels are prepared in the bulk scale, which is appropriate for local administrations. However, the high demand for nanosized and efficient systems for biomedical applications has led to the development of smaller DNA hydrogels that can be administered either orally (microscale), by inhalation (microscale) or systemically (nanoscale)²⁶⁸. This does not exclude the local administration of smaller DNA hydrogels as a bulk implant or as an injectable material, where they can promote a systemic effect *via* drug/gene delivery or an immunostimulatory response. This has opened the door for the further development of novel materials for *in situ* injectable, self-gelling and biodegradable DNA hydrogels¹³⁵. Here, the DNA hydrogel precursors can be injected simultaneously *in situ* and the hydrogel can form on the spot without the need for environmental manipulation (e.g. temperature and/or pH changes, enzymes, etc).

Overall, DNA hydrogels have been used for the release of small molecules such as drugs (cimetidine¹⁷⁹, CPT¹⁴⁶, insulin¹⁴⁶, nicardipine¹⁴⁹, coralyne¹³⁹, DOX, epirubicin (EPIR) and PTX^{88,144,161,165,168,182,216,241}), bigger molecules (proteins^{127,135,141,142,150,153,158,182,190,205,213,217,240,244,259} and DNA^{88,147,153,158,161,165,168,209,252}), nanoparticles (quantum dots (QD)^{141,210} and

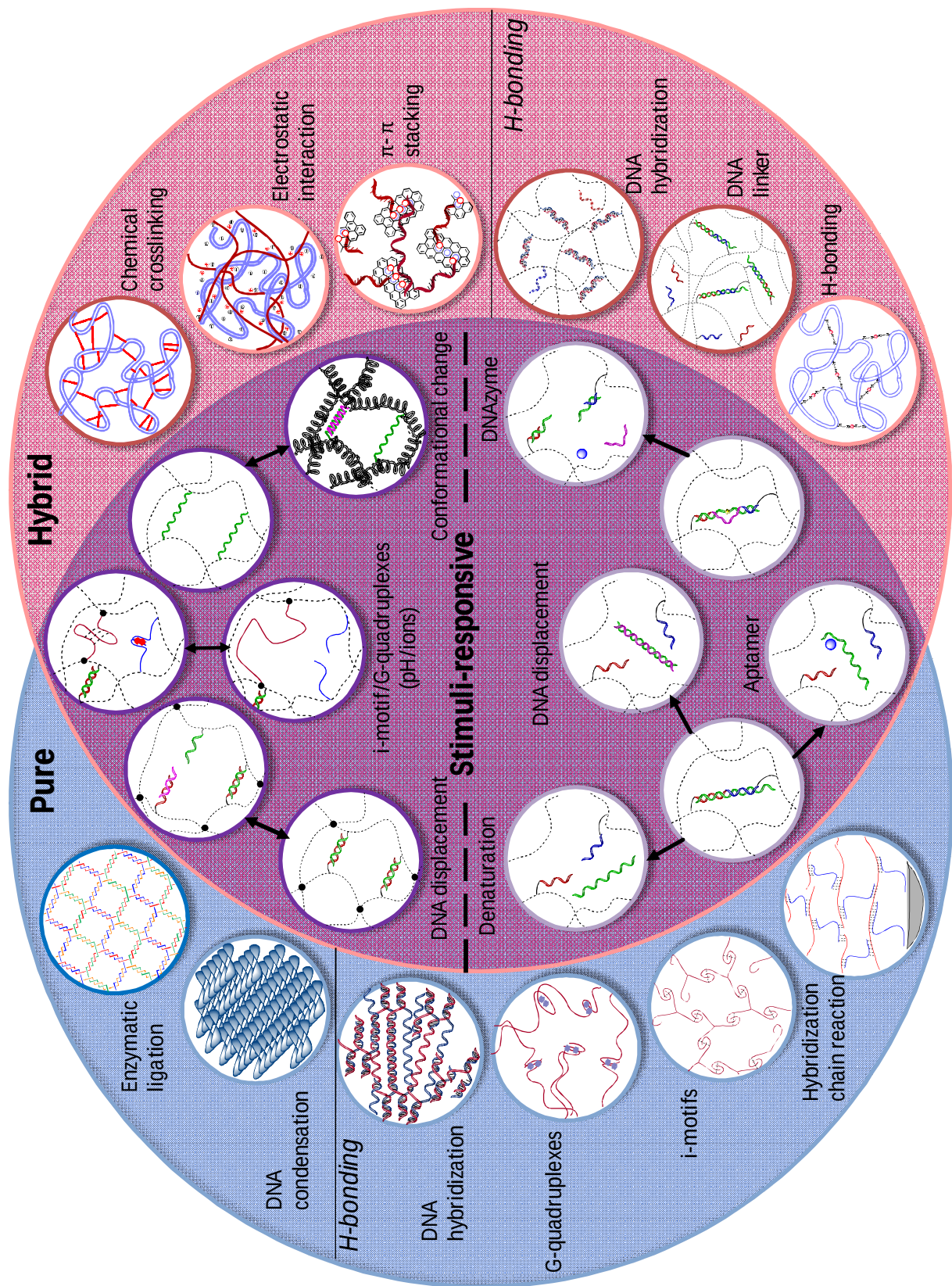
AuNPs^{127,129,151,182,193,211,216,218}) and microparticles (fluorescent-labeled melamine resin¹³⁷). Release of the loaded DNA hydrogel contents can be promoted by volume and phase transitions in the presence of the right triggers (e.g. pH, ions, specific ssDNA, etc.), where the released contents can be used for detection only (e.g. colorimetric AuNPs and fluorescence) or for an actual physical, chemical or biological function (e.g. an enzyme catalytic reaction vs. a cascade of enzyme reactions, where the products of the first enzyme trigger the next enzyme in the chain to convert its substrate into a detectable colored product^{127,132,142,190,203,213,218,219,251,259,269}).

DNA-based hydrogels as tridimensional scaffolds and cell-free protein synthesis platforms

Most DNA hydrogels occur in bulk form and are accordingly investigated in terms of their mechanical properties under different conditions^{195,208,212}. The possibility of modulating the mechanical properties of a hydrogel has allowed for the investigation of cell behaviour under dynamic mechanical conditions^{187,188,199,214}. From a biological and biomedical point of view, this is a highly interesting concept. Due to the biocompatibility and primary genetic function of DNA, DNA hydrogels have thus been prepared envisioning their use as tridimensional extracellular matrix-like cell scaffolds^{204,254} with self-healing properties^{125,136,254} that can be also used to explore single-cell interactions^{244,250} and to synthesize proteins cell-free^{124,239,243}. Actually, DNA hydrogels surrounded by a cell-membrane-like lipid layer have been compared to a synthetic nucleus^{236,237,245}. The systems developed were found to be capable of maintaining the genetic information in its interior, and when in the presence of the gene transcription and/or protein translation and respective starting materials, gave rise to mRNA and/or proteins.

As most DNA hydrogels are envisioned for biomedical applications, their cytocompatibility and their hemocompatibility (*i.e.* interaction with blood cells) is important. The latter factor needs to be considered when preparing the hydrogel itself (no harsh reagents or solvents) or when assessing its degradation products (not toxic) in order to ensure that no severe side effects such as coagulation exist. Some works have addressed this issue^{235,258}, performing hemocytocompatibility tests with great results, where the DNA hydrogels were even shown to be both hemostatic and hemocompatible.

The following graphical summary shows everything that has discussed up to now, in terms of the DNA type, methods used and stimuli-responsive behaviour of the most common DNA hydrogels developed to date.



3. DNA-based films

A film can be defined as a continuous thin layer of material(s), with its extension along two dimensions (2D) and with a limited thickness (3D)²⁷⁰. Moreover, a film is always in close contact with a substrate, either where no interactions are established (*i.e.* the substrate only serves as a template for cast films, wet spinning, etc.) or where some type of interaction is established (*e.g.* physical interactions through non-covalent bonds and/or chemical interactions through covalent bonds). Films can constitute an individual layer or multiple layers of a material or a combination of several materials, coating a specific substrate that can in turn be planar or colloidal in structure (Figure 37). These layers can be assembled from the nano- to the macro-scale, being categorized as ultrathin films (1-100 Å; *e.g.* monolayers), thin films (100-1000 Å; *e.g.* multilayers) and thick films (1000 Å-20 μm). Films can also be non-detachable or they can be detached simply by peeling it off the substrate, dissolving the substrate (*e.g.* colloidal substrates) or using the sacrificial layer approach²⁷¹.

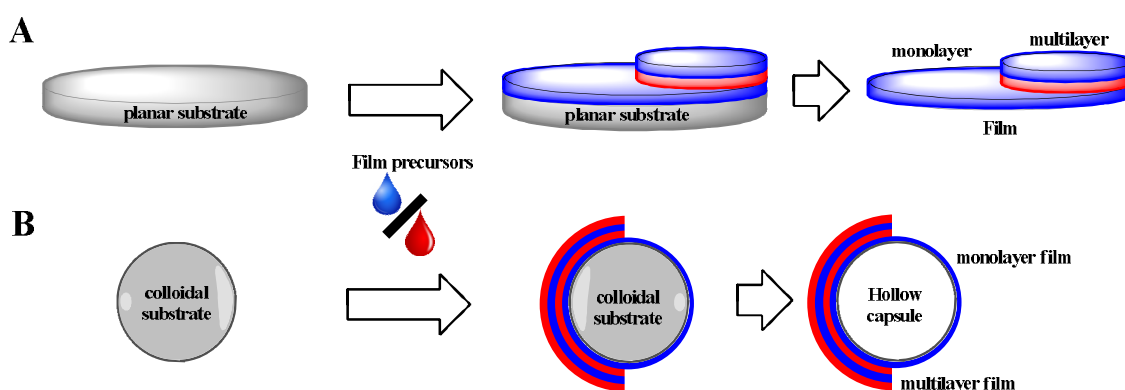


Figure 37 - Constitution of films on different types of surfaces. Mono- or multilayered film preparation (A) on a planar substrate with or without detachable/self-standing properties, and (B) in a colloidal substrate that is surface-modified or has a template function (*e.g.* to obtain hollow capsules).

Depending on the potential applications of film surface modifications, both the film and the surface may have an essential role. The substrate, on one hand, may just be a physical support/template to which the film will be deposited²⁷² or it may also have a specific functionality like signal detection (*e.g.* the use of a conductive substrate with charge transfer properties)²⁷³. The film, on the other hand, may confer special properties to the substrate that it initially does not possess, such as conductivity, targeted biomolecular recognition/detection and binding properties²⁷⁴.

Nowadays, in the materials science field, films can be constructed using various types of materials ranging from inorganic (*e.g.* metals)²⁷⁵ to organic (*e.g.* polymers)²⁷⁶. Different methodologies can also be used in their preparation, with more or less sophisticated equipment. Several deposition methods currently exist to produce films with different composition and

thickness. In fact, these methods can involve physical or chemical approaches (Table 1) that allow for the synthesis of films with controllable shape and thickness²⁷⁷. The method choice will mainly depend on the type of starting material (or precursor) to be used, as well as the type of film to be prepared and its desired thickness. In general, organic molecules and biomolecules require non-destructive physical and/or chemical methods, usually in the liquid phase. In terms of cost, chemical processes are considered to be the cheapest way to manufacture thin films. This is in contrast to the more costly physical vapor deposition (PVD) approach, which requires special and specific equipment.

Table 1 - Most commonly used physical and chemical techniques for thin film surface deposition and general representation of each category (images are examples of the techniques). Adapted from ²⁷⁷⁻²⁸⁰.

Thin Film Deposition Techniques				
Physical		Chemical		
Liquid-Phase Coating	Gas-Phase	Liquid-Phase	Gas-Phase	
Physical Vapor Deposition (PVD)		Chemical Bath Deposition (CBD)	Chemical Vapor Deposition (CVD)	
Spray-on Coating	Sputtering	Sol-Gel	Atmospheric Pressure CVD (APCVD)	
Spin-on Coating	Diode and Triode Sputtering	Plating (Electrochemical Deposition, Electroless, Anodization)	Atomic Layer Deposition (ALD)	
Painting	AC/RF and DC Sputtering	Liquid Phase Epitaxy (LPE)	Combustion CVD (CCVD)	
Doctor Blading	Reactive Sputtering	Spray Pyrolysis Technique (SPT) and Ultrasonic SPT	Hybrid Physical CVD (HPCVD)	
Dip Coating	Getter and Bias Sputtering	Arrested Precipitation Technique (APT)	Hot Filament CVD (HFCVD)	
Drop Coating	Ion Beam Sputtering (IBS)	Polymer Assisted Deposition (PAD)	Laser-Induced CVD (LCVD)	
	Magnetron Sputtering (RF, DC, HIPIMS, HiTUS, DMS)	Surface Functionalization/Grafting	Low Pressure (LPCVD)	
		Electrospinning	Metal-Organic CVD (MOCVD)	
		Electrophoretic Deposition	Photo-Initiated CVD (PICVD)	
		Dip Coating	Plasma-Enhanced CVD (PECVD)	
			Rapid Thermal CVD (RTCVD)	
			Vapor-Phase Epitaxy (VPE)	
			Thermal Oxidation	
			Thermal Nitridation	
			Thermal Polymerization	
			Oxidative CVD	

Another important factor to consider in this field is the constant need for miniaturization, which has in fact allowed for the continuous development of new materials and techniques to achieve ultrathin and thin films. Regardless of the scales attained, films may be considered an important piece of the world's economy, as they impact significantly on the evolution of modern technology in a wide range of sectors. From conceptualization of design to synthesis, film development is quite varied, being found in microelectronics, opto-electronics, optics, biomedicine, food industry, electricity, mechanics, magnetics, sensors, chemistry, energies, engineering etc²⁷⁹.

DNA-based films may have DNA included as one of the components of the film or, in some cases, as the only component of the film. Due to its biocompatibility, biodegradability, genetic information, as well as its physical and chemical properties (e.g. sequence recognition and charge transfer), DNA is considered a good starting material/precursor for film production to be used in different applications, such as gene delivery²⁸¹ and sensors²⁸². Also, dependent on their intended application, DNA films may be prepared using different DNA molecules whether of natural or synthetic origin.

Indeed, DNA-based films can be prepared either onto a substrate by physical adsorption or onto a previously applied layer through a process that is driven by non-covalent interactions including hydrogen bonds, π - π interactions, intercalations, groove binding, *van der Waals* interactions and hydrophobic interactions²⁸³. On the other hand, DNA can be chemically linked to a substrate or to a layer through covalent bonds. The main advantage of covalent bonds over non-covalent interactions is film stability. Covalent bonds are more stable and thus difficult to disrupt, whereas non-covalent bonds are less stable and are easily disrupted by changes in the conditions to which they are exposed (e.g. pH, ion concentration, external pH variation, reduction and oxidation reactions, competitive binding, charge shift, etc.)²⁸⁴. DNA films can also undergo degradation through hydrolytic or enzymatic process, or even reduction of disulfide bonds or they can also be destroyed mechanically or by temperature-induced transitions.

Although numerous published works about DNA-based films exist, one should bear in mind that the authors do not necessarily designate the assembled products as DNA films. Rather, the authors tend to refer to the DNA films (i) in terms such as DNA immobilization / deposition / assembly / adsorption / surface modification, (ii) as ultrathin / thin / layer / monolayer / multilayers / capsule, (iii) on electrode / surface / particles, or (iii) for conductivity / charge transfer / DNA hybridization / sensor applications. Thus, as is evident, the techniques used to prepare DNA-based films are diverse, ranging from physical to chemical approaches. Currently, the methods most commonly used to obtain DNA films include the Langmuir-Blodgett technique (LB), covalent surface functionalisation (including Self-Assembled Monolayers (SAMs)), Layer-by-Layer self-assembly (LbL), solution casting techniques and wet-spinning methods.

In the next section, the concepts and techniques used to develop DNA films will be comprehensively discussed. This will be accompanied with examples of the work developed in the field over the years. Accordingly, the DNA films will also be categorized according to the technique used to prepare them.

3.1. Langmuir-Blodgett (LB) technique

The Langmuir-Blodgett (LB) technique is based on the physical self-assembly of molecules, that is, molecules in a disordered system become organized, forming a homogenous and organized structure due to non-covalent interactions²⁸⁵. The formed structure, designated as a LB monolayer or film, shows a high degree of organization and has been described as a continuous and quasi-crystalline film²⁷⁴. Broadly defined as water-insoluble amphiphile monolayers, LB films can be prepared through the use of volatile organic solvents that are initially spread in an aqueous phase and that are then later transferred onto a solid substrate²⁸⁵. Since the film precursors are water-insoluble amphiphiles (made up of hydrophilic and hydrophobic groups), when added to a bath, they tend to locate at the interface. The particularity of this assembly at the bath interface allows for the formation of an ordered LB monolayer with variable molecular density.

In more detail, this technique involves the addition of the amphiphilic film precursors to a bath in a trough apparatus that has lateral moving barriers (Figure 38, A). After the solvent evaporates, the lateral barriers begin to move, varying the pressure on the monolayer accordingly. The movement of the barriers either inwards or outwards will lead to a respective increase or decrease in the molecular density of the film²⁸⁶. The transfer of the film onto the substrate always occurs using the dipping technique. The process here may occur either vertically (corresponding to the more conventional LB method) or horizontally (corresponding to Langmuir-Schaefer's (LS) method) by either emersion (retraction or upstroke) and/or immersion (dipping or downstroke) substrate displacement^{276,286}.

The organization of the LB film and its transfer to a solid surface will depend on the experimental conditions used, such as the pressure on the compaction of the monolayer (affects film density), the dipping speed (affects film stability), the precursor concentrations, the nature of the substrate and the temperature values²⁸⁶. For example, if the dipping speed of the substrate is higher than the monolayer adsorption, the film is unstable due to low adsorption. This is referred to as non-reactive deposition. In contrast, if the monolayer molecules spontaneously adsorb to the substrate through a process of reactive deposition, the film becomes stable.

LB films can be constructed in multilayers through successive dipping. The different monolayers in a multilayered LB film can assume one of three possible layouts (Figure 38, B)²⁸⁶. In the X-type layout, the monolayer only transfers onto the hydrophobic surface at immersion, with hydrophobic groups always facing the surface. In the case of the Y-type layout, the monolayer transfers at both up and down stroke, with alternating groups facing the surface. The Z-type layout involves monolayer transfer onto the hydrophilic surface only at upstroke, with hydrophilic groups facing the surface. These deposition layouts depend on various conditions such as temperature, surface pressure, pH, ion concentration and especially on how strong or weak the hydrophobicity and hydrophilicity of the monolayer precursors are.

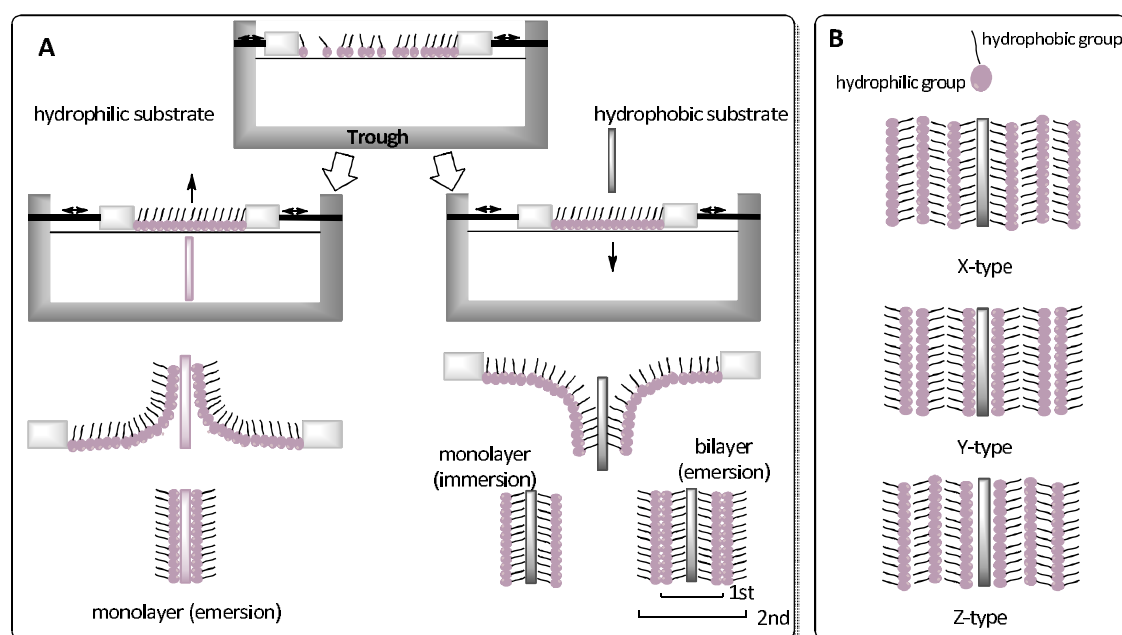


Figure 38 - LB thin film deposition technique. (A) Preparation and transfer of LB film onto hydrophilic and hydrophobic surfaces. (B) Types of hydrophobic surface deposition obtained by repeat immersion cycles, with example of the multilayer types formed.

The use of the LB technique for film formation is considered to be an expensive and impractical process. Contributing to this are factors including the environmental conditions used (e.g. atmosphere, temperature, humidity and dust), the purity of the solutions used (e.g. precursors, solvents, bath, etc.) and the cleanliness of the substrate, all of which are of extreme importance to obtain a stable, uniform and contaminant-free LB film. Nevertheless, several advantages to the use of this technique exist. The generation of LB films with highly ordered and oriented molecular arrangements and controllable thickness make these materials attractive for use in nonlinear optics, piezoelectrics, pyroelectrics, electro-opticals, molecular engineering devices, sensors, conductors, semiconductors, solar cells, evaporation control, gas exchanges, chemical reactions and binding

affinity characterization²⁸⁷. In addition to being used for the deposition of organized molecules, this technique has also allowed for the deposition of nanoparticles, nanofibres, nanotubes and nanowires^{288–291}. Bilayer deposition resembling the cell membrane has also been reported, with biomacromolecules such as cell receptors, channel proteins or enzymes being incorporated into or immobilized onto the bilayer surface allowing for interaction and binding mechanism studies, among others²⁹².

DNA is a water soluble molecule and as such, may appear to be an unsuitable precursor when preparing films using the LB technique. Although this molecule does possess a hydrophilic backbone and a hydrophobic interior, its inherent hydrophobicity is insufficiently strong to allow for its solubility in organic solvents (e.g. DNA precipitates in ethanol which is a polar solvent). Combination of DNA with amphiphilic molecules in the LB technique has however been used as an alternate strategy. Here, after dissolution of DNA on the lower aqueous subphase, the cationic amphiphilic molecules were deposited onto its surface. At the interface, an exchange of the DNA counterions for cationic lipids followed, resulting in the electrostatic interaction between the DNA phosphate groups and the lipid cationic headgroups. Thereafter, the formed DNA/cationic amphiphile monolayer could be transferred using the standard dipping procedure (Figure 39).

To our knowledge, Erokhin *et al.*²⁹³ were the first to use the LB technique in combination with nucleic acids in 1992. Their results showed the adsorption of small DNA fragments onto the surface of an amphiphile layer that led to the eventual formation of a sandwich-like film, where a DNA layer was sandwiched between the two amphiphile monolayers. Five years later, a very interesting study reported the application of the same type of film to construct a DNA hybridization sensor based on nanogravimetric balance²⁹⁴. In this work, single stranded plasmid DNA (ss-pDNA) molecules were incorporated between two amphiphile layers. After the incremental addition of complementary DNA probes to the ss-pDNA-containing films, hybridization occurred. The resultant variation in mass during the hybridization process could be detected by a quartz nanobalance.

In 1996, Okahata *et al.*²⁹⁵ also saw the potential of combining the LB technique with DNA to create DNA films, knowing in advance the charge transfer properties of DNA and that the DNA molecules would be ordered and aligned using this technique. Indeed, the orientation of the DNA molecules in the transferred films was dependent on many factors. In brief, the DNA strands were aligned a) when the monolayer was compacted (as was expected), b) when vertically transferred onto the substrate (this was influenced by the flow; in contrast, horizontal dipping showed a random arrangement), and c) DNA was previously mixed with intercalators. In the latter case, the alignment of the DNA strands was a result of the increase in rigidity of DNA when in the DNA/intercalator complex, which in turn made orientation easier. The combination of the above factors gave rise to

conductive films with charge transfer, property which was attributable to the stacked bp and the presence of efficient DNA intercalator adsorbers^{295,296}.

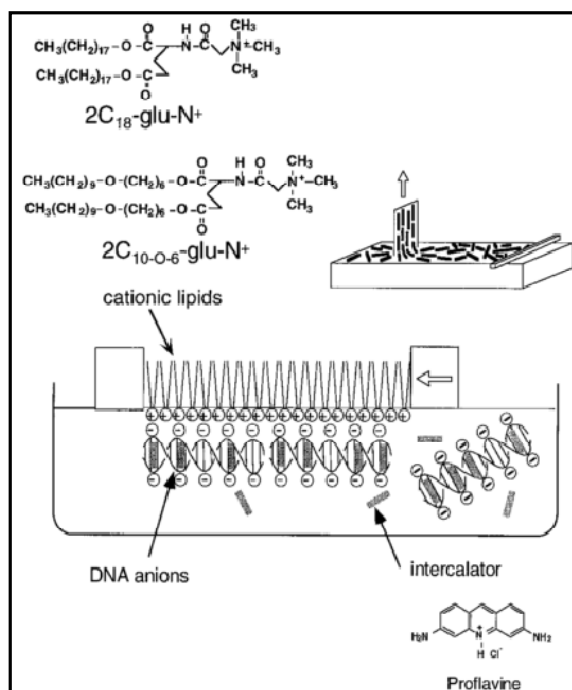


Figure 39 - DNA-oriented LB film prepared using a DNA/intercalator complex and a monolayer made up of cationic amphiphiles²⁹⁵. Adapted/reprinted with permission from ref. 295, Copyright © 1996 American Chemical Society.

In the following years, works developed around DNA films produced by the same LB strategy envisioned their use in applications such as binding of intercalators, electron charge transfer, binding of ions, DNA/amphiphile interaction studies, affinity for protein recognition, enzymatic reaction, DNA transcription, and in particular, DNA hybridization sensors^{294,297-301}. An interesting study presented by Bhaumik *et al.*²⁹⁸ used long dsDNA immobilized on a monolayer of Zn-arachidate formed by the LB technique. Since DNA has affinity towards Zn(II) through coordination, it was possible to form a layer of DNA over the monolayer of Zn-arachidate. In the film, the DNA strands bundled up into thread-like structures and aligned along the pull direction of the substrate. ssDNA and dsDNA were used to study strand hybridization and enzyme accessibility, respectively. Although bundled up in the monolayer, DNA was still available for enzymatic digestion by DNase I. It was found that just 5 minutes of digestion were sufficient for the strand length to decrease from 1.6 - 1.7 μm to 0.6 - 0.7 μm . For the authors, these results prompted further studies in which DNA-RNA polymerase interactions in the LB film were explored and transcription experiments were performed^{302,303}.

Although not as common as other techniques to produce DNA films, the LB approach has advantages such as the preparation of highly organized and compacted layers. It is however an expensive and elaborate technique that can only be applied to planar substrates.

3.2. Covalent substrate functionalisation

Ultrathin films can be prepared over a surface by chemical functionalisation which involves the establishment of covalent bonds between the surface and the chemical entities that will make part of the films. The covalent immobilization of DNA onto any surface leads to films with greater stability, being an important feature in reusable devices such as sensors and biosensors³⁰⁴. Preparation of this film type usually involves the use of small DNA molecules, which leads to organized films with well-defined layers.

Thus, the substrate needs to present reactive functional groups that behave as anchors at its surface (Figure 44), such that a reaction may occur that will then allow for the immobilization of DNA molecules through covalent bonds. The substrates can suffer silanization (in the case of silicon or metal oxides), etching by chemical and physical methods such as plasma or acids treatments (exposure of internal groups), oxidation (to give rise to hydroxyl, carbonyl or carboxyl groups), amination, or thiolation. Afterwards, DNA can be immobilized in the substrate through suitable chemical reactions that may use spacers and/or linkers. A chemisorption process may also be used to covalently link DNA into a surface, like happens when self-assembled monolayers (SAMs) are formed. SAMs constitute a very special (and important) case of molecule organization at a surface and, due to this reason, they will be first addressed.

DNA Self-Assembled Monolayers (DNA SAMs)

The process involved in the preparation of SAMs is based on the spontaneous formation of an active surfactant monolayer on a specific substrate through covalent bonds. The particularity of this type of self-assembly is that the monolayer component is composed of three main parts, namely, a headgroup, an alkyl chain spacer and a terminal group, each of which plays an important role in monolayer formation (Figure 40).

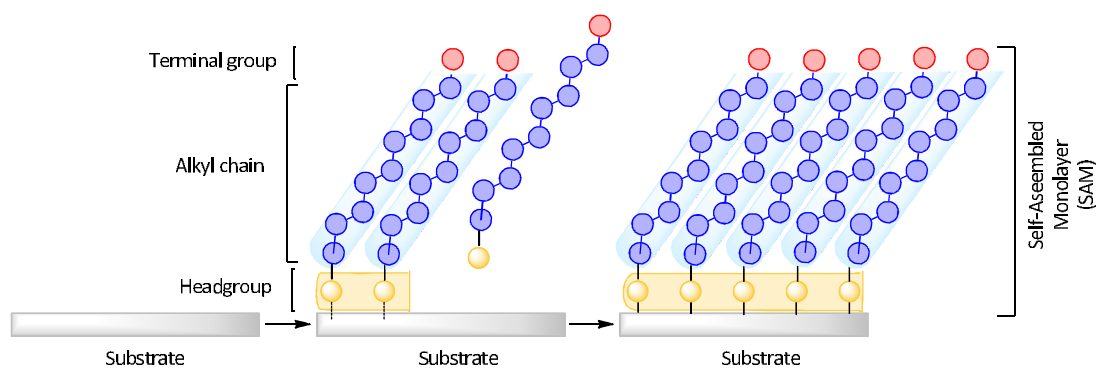


Figure 40 - Self-assembled monolayer (SAM). The headgroup is spontaneously chemisorbed onto the substrate, the hydrophobic spacer is responsible for the organization of the monolayer and the terminal group allow for the conjugation of other molecules or the assembly of following multilayers (e.g. a DNA molecule).

In SAM, the headgroup, which will depend on the substrate type and its functionalisation, can correspond to one of many functional groups. Oxide (e.g. hydroxyl groups), germanium or diamond substrate surfaces are usually combined with either alkyltrichlorosilyl, methoxysilyl or olefin through strong covalent bonds (e.g. Si-O)^{305,306}. Gallium arsenide (GaAs), cadmium selenide (CdSe), indium(III) oxide (In₂O₃), Pt, Ag, Cu and Au surfaces can be combined with alkanethiols, disulfides (S-S), selenol (SeH) and isocyanide (-NC) forming polar covalent bonds (e.g. Au-S). During the chemisorption process, the headgroups on the molecules occupy every binding site available on the substrate before forming bonds through covalent interactions. Regarding the spacer component of SAMs, *van der Waals* interactions between the alkyl chains (electrostatic interactions in the case of modified alkyl chains) maintain them in close contact with each other resulting in the organized and closely packed assembly of the monolayer. The terminal group may be a methyl or a functionalisable group that will either allow for the layering of a second SAM or be chemically modified with a desired molecule³⁰⁷, for example DNA.

The organization and density of the monolayer will depend greatly on the bulkiness of the headgroup and the alkyl chain length³⁰⁸. The bulky headgroups create space at the base of the monolayer and consequently between adjacent alkyl chains and terminal substituents. As a result, a decrease in molecular density and an increase in conformational flexibility will be observed in the monolayer. The probability of finding defects known as pinholes on such monolayers is high, but in very low concentrations and where the molecular density is lower. Long alkyl chains, on the other hand, are less flexible relative to shorter alkyl chains in the space defined by the headgroup and as such lead to the formation of densely packed monolayers³⁰⁹.

The most common and simpler examples of DNA SAMs use alkanethiol-modified DNA molecules (DNA constitutes the terminal group of the SAM components) which directly self-assemble

in a surface giving highly organized and densely packed SAM structures. Indeed, numerous studies about DNA SAMs can be found in the literature of which some representative examples will be presented next.

In the late 90s, Herne *et al.*³¹⁰ and Levicky *et al.*³¹¹ immobilized alkenethiol-modified ssDNA onto gold surfaces via S-Au covalent bonds. In order to avoid non-specific adsorption of DNA onto the Au surface, a mixed SAM was prepared using modified DNA and a thiol spacer (6-mercapto-1-hexanol (MCH))³¹⁰ (Figure 41). Salt concentration was found to be an important factor in DNA adsorption, with low salt concentrations resulting in low DNA coverage of the Au surface. The surface density of immobilized DNA (so-called probe density) was later quantified using an electrochemical method developed by Steel *et al.*³¹², where the resolution of the technique allowed for ssDNA and dsDNA (after hybridization) quantification. The DNA coverage of the Au surface was also found to be concentration and oligonucleotide length dependent³¹³. The short DNA probes were reported to adopt an extended conformation, perpendicular to the surface, thus allowing for a higher probe surface density. In contrast, the long DNA probes showed a polymer-like behaviour where their disordered arrangement prevented other DNA probes from being immobilized.

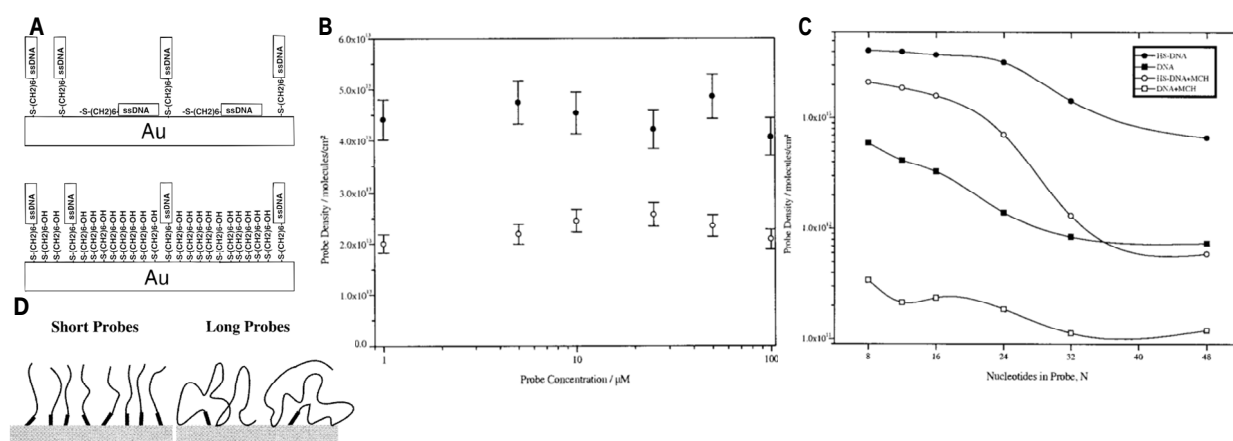


Figure 41 - DNA self-assembled monolayer (SAM). (A) Schematic of ssDNA immobilized onto a Au surface (top) and ssDNA/MCH adsorbed onto a Au surface (bottom)³¹⁰. (B) Probe density of oligonucleotide on Au surface as a function of deposition concentration, before (filled circle) and after (open circle) MCH treatment³¹³. (C) Probe density of oligonucleotide on Au surface as a function of thiol modification and oligonucleotide length, before (filled circle) and after (open circle) MCH treatment³¹³. (D) ssDNA configurations as a function of oligonucleotide length, where short probes present an extended conformation in contrast with longer probes³¹³. Adapted/reprinted with permission from ref. 310, Copyright © 1997 American Chemical Society and ref. 313, Copyright © 2000 Elsevier

While Herne and Levicky determined DNA surface coverage using electrochemical methods, Demers *et al.*³¹⁴ used a fluorescence-based method involving the incorporation of fluorescent-labelled DNA to the monolayer. Once again, DNA (hexanethiol 12-mer oligonucleotides were used) coverage of the Au surface was concluded to increase with increasing salt concentration and was

found to be dependent on the composition and length of the DNA sequence. The insertion of an oligonucleotide spacer into the probe DNA sequence, was also shown to influence the extent of DNA surface coverage. For example, a 20-mer polyA spacer was reported to partially block the Au binding sites by behaving in a similar manner to the previously mentioned long polymer-like DNA probes. A 20-mer polyT spacer, on the other hand, was in a more perpendicular extended conformation relative to the Au surface thus promoting higher surface coverage. Using the fluorescence-based method, the determination of the hybridization efficiency of the oligonucleotide probes as a function of substrate and spacer type was also evaluated. The hybridization efficiency of the oligonucleotides was 33% and 4% in the planar (Au surface) and colloidal (AuNP surface) substrates, respectively. In the case of the AuNP substrate, the results obtained reflect the limited access of the oligonucleotides for hybridization due to steric hindrance and electrostatic repulsive interactions. When the monolayer was assembled using spacer-containing oligonucleotide probes, the hybridization efficiency increased to 44% in the AuNP substrate indicating that a distance further away from the substrate surface allowed for more space for strand hybridization to occur. A conclusion that can be taken from this work is that a densely packed SAM with a high DNA density will form a well-organized structure. Strand hybridization efficiency however, will be compromised if the space available for the hybridizing DNA strand to approach and interact with the DNA probe on the substrate surface is limited. The only way to increase hybridization efficiency is to have control over substrate surface coverage and DNA density.

Analogous to the previous studies, Cynthia Bamdad³¹⁵ also prepared DNA SAMs using Au as the substrate surface. In this case, the SAMs were prepared using a mix of DNA-triethylene glycol alkanethiols and inert triethylene glycol-alkanethiols (used to prevent the nonspecific binding of proteins). Of particular interest in this work, were the DNA duplexes formed after hybridizing complementary oligonucleotides possessing sticky ends to the oligonucleotide probes on the SAM surface. Protein binding studies showed that the resultant dsDNA duplexes with exposed sticky ends were stabilized by the ligase enzyme due to the formation of phosphodiester bonds that allowed for the closing of the nicks.

Georgiadis *et al.*^{316–319} also studied the kinetics behind SAM assembly and oligonucleotide hybridization (complete or mismatched) by surface plasmon resonance (SPR). Kinetic parameters including adsorption, desorption and diffusion were all assessed. Overall, the studies showed that the kinetics of oligonucleotide hybridization was not only influenced by the presence of mismatches but also by the location at which hybridization occurs³¹⁷, with mismatches at the first base pairs resulting in less stable duplexes.

Alternate approaches from the traditional method have also been designed to achieve DNA SAMs. In the approach followed by Satjapipat *et al.*³²⁰ for example, a Au surface was primarily

modified with a mixed SAM composed of either mercaptopropionic acid (MPA)/hexanethiol (HT) or MPA/mercaptohexanol (MCH). Thereafter, a selective desorption of MPA led to the exposure of the Au substrate to which alkenethiol-modified DNA was then chemisorbed (Figure 42, A). It was found that the size of the exposed Au surface after desorption could be controlled by the MPA/HT ratio. Moreover, the DNA surface density began to increase when the size of the exposed Au surface was comparable to that of the alkenethiol-DNA molecule. The hybridization efficiency in the mixed SAMs was 95.5% relative to the 19.6% hybridization efficiency in the pure alkenethiol-DNA SAMs. A very interesting strategy later adopted by Nakamura *et al.*³²¹ involved the preparation of branched DNA SAMs which were clearly shown to play a role in increasing SAM-based DNA biosensor sensitivity, efficiency and reproducibility (Figure 42, B). An added advantage to the developed system was that the branched DNA probe allowed for the possibility to detect several different target DNA sequences.

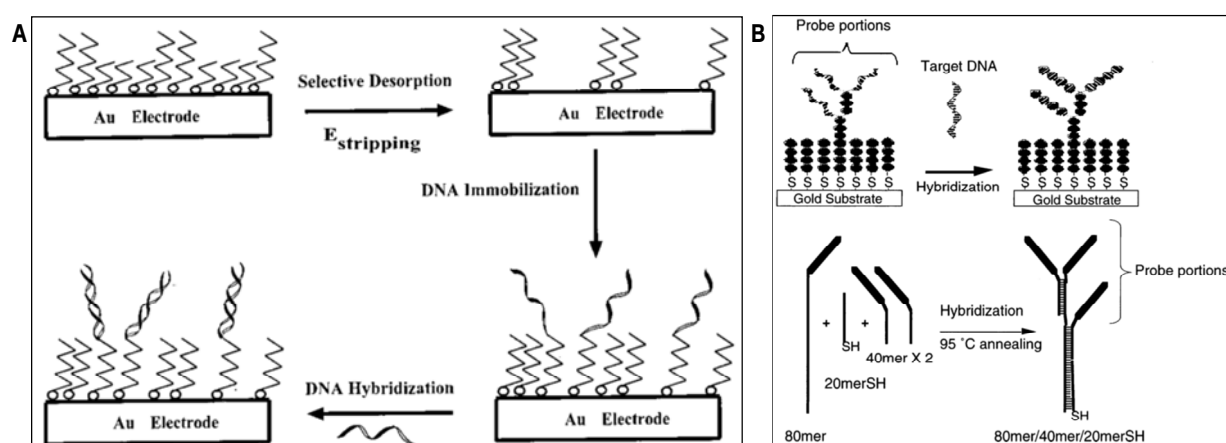


Figure 42 - DNA SAM with mixed alkane chains. (A) Chemisorption of mixed SAM, followed by desorption of shorter alkanethiols and then alkanethiol DNA immobilization for hybridization purposes³²⁰. (B) Hybridization to branched DNA SAM with three probe portions³²¹. Adapted/reprinted with permission from ref. 320, Copyright © 2001 American Chemical Society. and ref. 321, Copyright © 2003 American Chemical Society.

Takeishi *et al.*³²² proceeded to study the electrochemical release of DNA from Au surfaces as a function of different DNA linker types through the application of a negative voltage. DNA release was dependent not only on the voltage duration, but also on the linker type. Based on these results, the local delivery of DNA into cells by electrochemical DNA desorption was suggested. Electrochemical desorption was also performed by Sánchez-Pomales *et al.*³²³ to control the coverage of SAMs on Au surfaces and to enhance DNA hybridization efficiency. The understanding of the organization and orientation of DNA SAMs under different environmental conditions was also explored, as this type of information is important for the future development of devices (e.g. biosensors in aqueous solutions)^{324,325}. A very interesting work that stands out was done by Rant *et al.*³²⁶, where a "switch" DNA was used to simultaneously detect and size target proteins. The method developed here is based on the protein binding process promoting an electrically driven

conformation switching of DNA layers that were previously self-assembled on a Au surface. IgG antibodies and antibody fragments could be detected in quantities of less than 10^{-18} mol on the surface of these sensors.

In fact, DNA SAMs have been produced for several applications, the most important ones involving immobilized ssDNA probe used for the detection of DNA hybridization (e.g. DNA sensors, DNA microarrays, etc)^{327,328}. On the other hand, researchers have taken advantage of the base π stack of dsDNA and its conductivity abilities. This has been used to examine electron transfer^{329,330} when DNA interacts with other moieties such as intercalators, proteins and DNA, as well as perturbations within the base pairs stacking³³¹.

An important issue was highlighted by Petrovykh *et al.*³³² that is if hydrocarbon chains are absent in DNA monolayers, they cannot be called SAMs as they display very few properties typical of SAMs (Figure 43). In a typical SAM, *van der Waals* forces govern the tight and compact organization of the monolayer, while strong electrostatic repulsion exists among the DNA strands in the DNA monolayers. Moreover, DNA strands are more flexible than alkyl chains. In the study done by Petrovykh and coworkers applying several spectroscopy techniques, short and long thiol-modified ssDNA molecules were used to understand their adopted structure on the Au surface after chemisorption. It was demonstrated that non-modified DNA (dT₅) would preferentially adsorb onto the Au surface through the nucleobase (π interactions). On the other hand, the short thiol-DNA ((dT₅)-SH) would somehow be organized. This organization was attributed, not to hydrogen bonding between the strands, but to base stacking. The longer thiol DNA strands ((dT₂₅)-SH) showed a polyelectrolyte brush orientation due to higher strand flexibility. So, following the opinion of this researcher, in this review, DNA monolayers based on DNA-SH instead of DNA-hydrocarbon-SH will not be classified as SAMs.

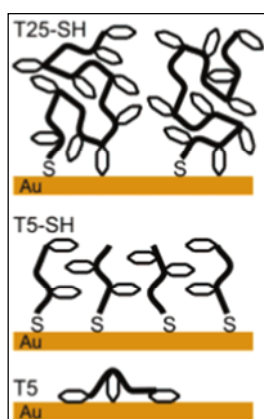


Figure 43 - Different length DNA strand orientation on Au surface. DNA molecules tend to interact with the gold surface through the nucleobases, as thiol-modified DNA molecules interact are chemisorbed to the surface by the thiol group³³². Adapted/reprinted with permission from ref. 332, Copyright © 2006 American Chemical Society.

Reaction between chemical groups present in the DNA and in the substrate

Regarding the covalent attachment of DNA onto a surface and beyond SAMs, researchers have made use of several diverse strategies involving chemical reactions. Indeed, DNA monolayers can be prepared through the reaction between functional groups at the surface and functional groups present in DNA (usually 5'end modified ssDNA is used). Strategies have ranged from thiol-modified DNA (without hydrocarbon chains otherwise SAMs would be formed) directly immobilized on gold surfaces by chemisorption³³³ to the immobilization of thiol-modified DNA onto a silanized amine terminated surface through the use of heterobifunctional crosslinkers that are thiol and amine reactive (Figure 44)^{273,334–337}.

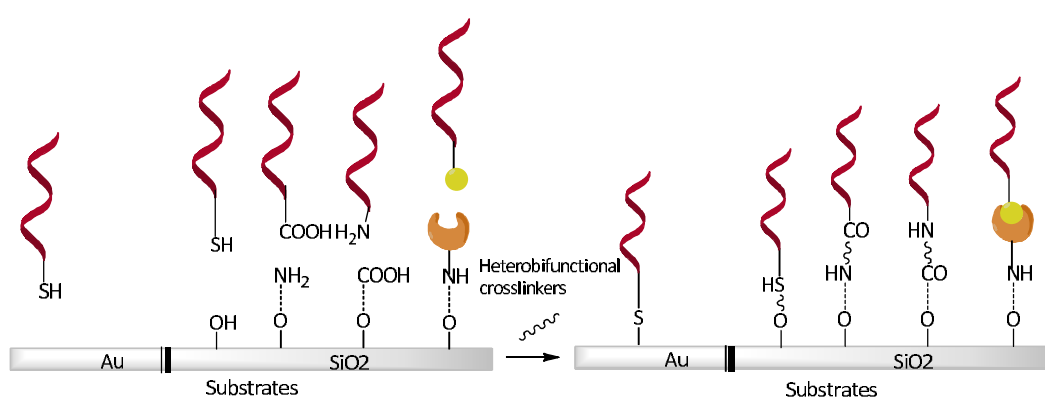


Figure 44 - DNA monolayers. 5'end-modified DNA molecules are directly and covalently linked to a Au (spontaneous, in one step) or functionalised SiO₂ surface, by means of homo- (not shown) or heterobifunctional crosslinkers (in multi-step).

As an example, DNA has been linked to a hybrid film made up of a CHI/Fe₂O₃ cast layer with leaf-like Fe₂O₃ microparticles through the use of a terephthalaldehyde (TPA) crosslinker³³⁸ and also to a diamond film using a thymidine linker³³⁹. In a study by Chrisey *et al.*³³⁴, surface functionalisation, crosslinker type and solvent used, as well as thermal stability and wettability, were all assessed in terms of their influence on DNA immobilization by covalent attachment (Figure 45, A). It was found that relative to chemisorption, the covalent attachment of DNA to a film was more stable and allowed for the control of DNA immobilization in applications where patterning, high salt concentration or elevated temperatures were required.

Okahata *et al.*³⁴⁰ prepared DNA monolayers based on the avidin-biotin biospecific interaction (Figure 45, B). Briefly, a carboxylic acid-modified gold surface (in a QCM) was activated by the water-soluble crosslinker 1-ethyl-3-(3-(dimethylaminopropyl)carbodiimide (EDC) to incorporate an avidin moiety via amide bond. Thereafter, biotin-modified DNA self-assembled with the prepared surface through avidin/biotin interactions leading to the formation of a stable complex. Hybridization

kinetics was evaluated under different ion strengths and temperatures. It was shown that hybridization of the target DNA to the probe biotin-DNA was practically inexistant in the absence of NaCl and increased as ionic strength increased. This was probably due to the phosphate shielding effect by the ions and duplex stabilization, which decreased the electrostatic repulsion between DNA strands. Regarding the temperature studies, at a higher temperature (30°C) the hybridization process was observed to be fast (1 min) but inefficient (10%). When applying a low temperature (10°C), on the other hand, hybridization was observed to occur slowly (1 day) but more efficiently (100%). With an increase in temperature, the association constant (K_a) increased, being influenced by the dissociation rate constant (k_{-1}) and not the binding rate constant (k_1). Also, as the DNA strand length increased, an increase in K_a and k_1 and a decrease in k_{-1} was revealed. The presence of a mismatching base in the target oligonucleotide sequence led to a decrease in k_1 and an increase in k_{-1} , as would be expected, since DNA does not have the same affinity here as it does for a completely complementary strand.

A step-by-step DNA film prepared using a different strategy was presented by Wang *et al.*³⁴¹ (Figure 45, C). To a cysteamine SAM-modified gold electrode, AuNPs were further self-assembled leading to an increase in the electrode surface area. This was followed by the immobilization of thiol-modified ssDNA targets onto the newly fabricated electrode. Then, DNA immobilized onto silver nanoparticles (AgNPs) was then used to detect hybridization of the target DNA. More specifically, after hybridization of the target ssDNA immobilized on the gold electrode with the silver-labeled DNA probe, a rinsing step followed to remove any unhybridized DNA probes. The AgNPs immobilized on the gold electrode *via* hybridization were then dissolved by oxidation using KBr and KNO₃. The resultant reaction led to the production of [AgBr₂]⁻, which is easily detectable by anodic stripping voltammetry (ASV). Thus, using ASV, the amount of [AgBr₂]⁻ produced could then be indirectly correlated with the amount of DNA hybridized.

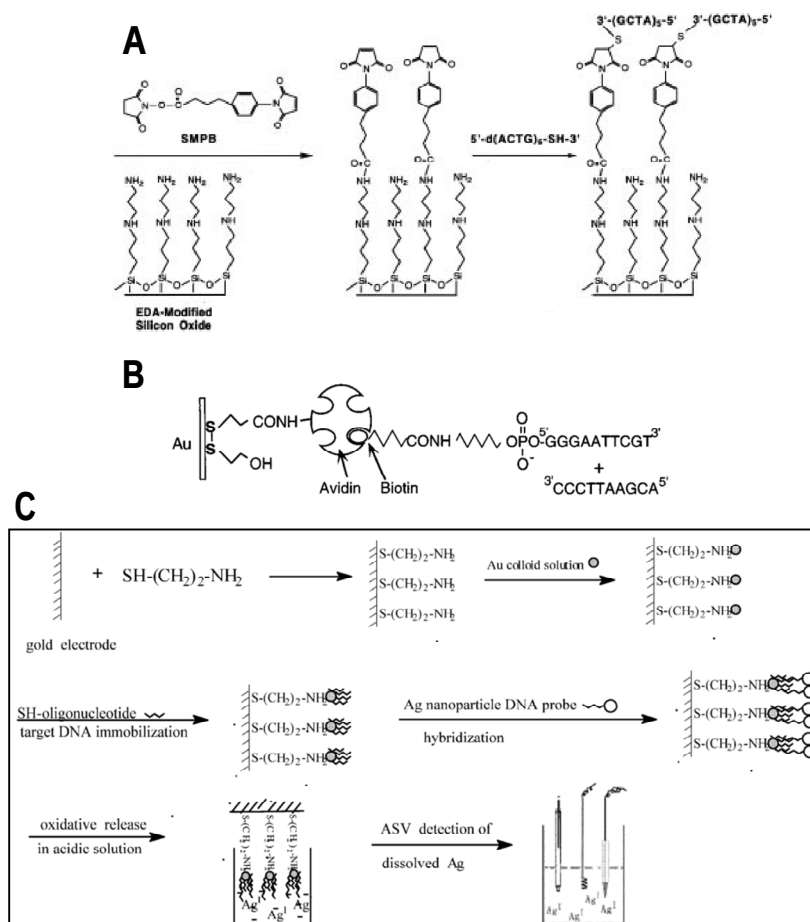


Figure 45 - Multi-step DNA monolayer preparation. (A) Thiol-modified DNA immobilized via a heterobifunctional crosslinker³³⁴. (B) DNA immobilization via avidin-biotin biospecific interaction³⁴⁰. (C) Target DNA immobilization onto gold nanoparticles, in turn immobilized onto a gold electrode. Hybridization was detected using a silver labeled DNA probe, where silver was dissolved and detected by ASV³⁴¹. Adapted/reprinted with permission from ref. 334, Copyright © 1996 Oxford University Press, ref. 340, Copyright © 1998 American Chemical Society, and ref. 341, Copyright © 2003 Elsevier.

Multi-step DNA monolayer strategies similar to the ones described above have also been widely applied to combine DNA with polymers to form DNA films. One such example involves substrate functionalisation, followed by further reaction with dendrimers on its surface through covalent linkage and final dendrimer functionalisation to then be used for the immobilization of DNA molecules^{342–346}. Dendrimers are highly organized and hyperbranched polymers, with well-defined molecular weight and with layers called generations³⁴⁷. At their periphery, dendrimers present multiple surface terminal groups that may be functionalised as desired. Several dendrimer types exist, but the most well known are the poly(amido)amine (PAMAM) dendrimers. As stated before, the highly compacted layer of DNA SAMs decreases DNA hybridization efficiency due to steric effects. Moreover, the preparation of DNA monolayers using spacers or linkers offers some flexibility and provides some space for hybridization to occur. Thus, the inclusion of dendritic polymers such as the PAMAM dendrimers into this type of DNA films comes as a result of their high amino-modified DNA

immobilization efficiency, great stability and large number of reactive binding sites for both substrate and DNA. Benters *et al.*³⁴² showed that DNA microarrays prepared using PAMAM dendrimers presented an increased signal intensity, thus improving the sensitivity of hybridization analysis. The same was observed by other authors in similar studies^{343–345}, where incorporation of dendrimers into the DNA films not only led to an increase in sensitivity and selectivity in the detection of DNA hybridization but also the ability to distinguish between DNA molecules with and without mismatch (Figure 46). Later, Zhang and coworkers³⁴⁶ prepared dendrimer-DNA monolayer films composed of thrombin-binding aptamers that were used to detect and quantify thrombin binding (Figure 46, D). The developed "aptasensor" revealed favorable regeneration, selectivity and stability in biological fluids.

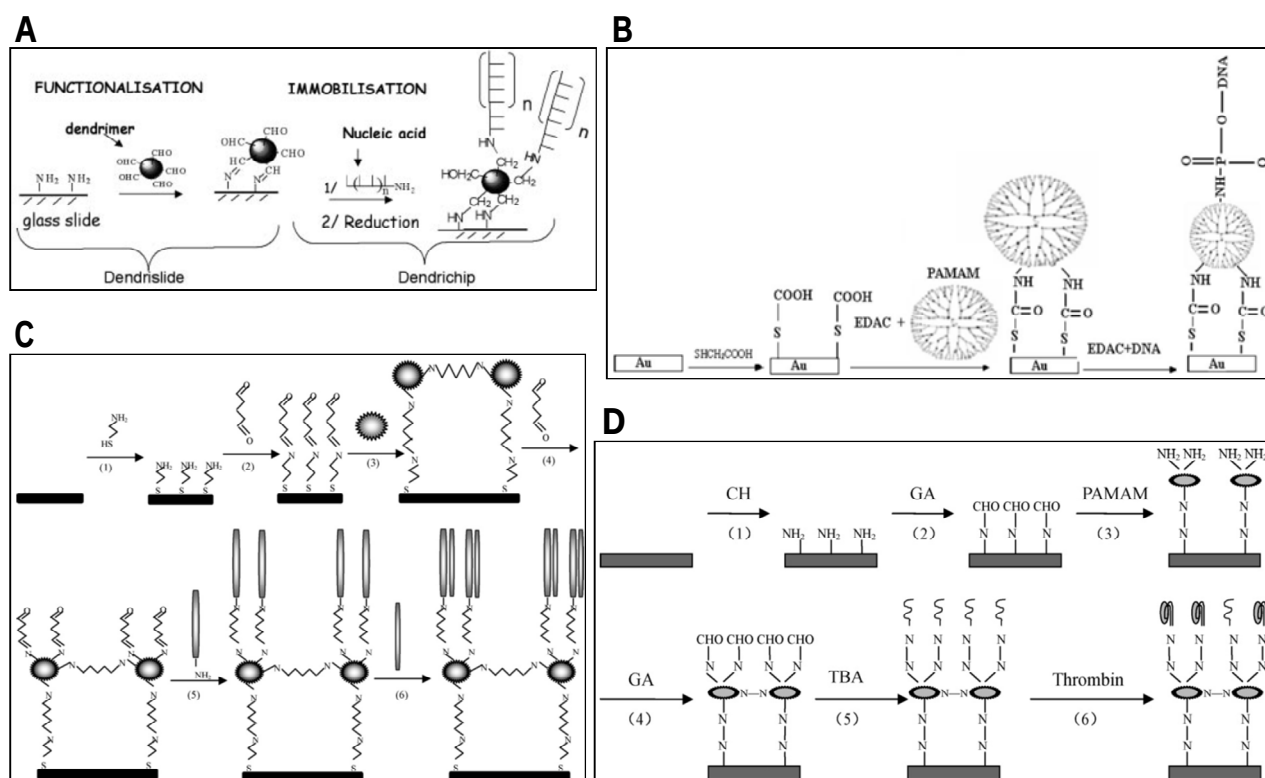


Figure 46 - Dendrimer-based DNA monolayer films. (A) Functionalisation of glass surface for dendrimer immobilization by covalent linkage, followed by DNA binding to the dendrimer surface³⁴³. (B) Conjugation of ssDNA onto PAMAM dendrimer previously linked to Au electrodes³⁴⁴. (C) Modification of gold surfaces first with a 2-aminoethanethiol (AET) SAM and then a glutaraldehyde (GA) spacer, followed by the further addition of dendrimer/GA as a spacer/linker and finally ssDNA probe immobilization³⁴⁵. (D) The same type of modification was performed, using as the DNA probe a thrombin-binding aptamer (TBA)³⁴⁶. Adapted/reprinted with permission from ref. 343, Copyright © 2003 Oxford University Press, ref. 344, Copyright © 2006 by John Wiley & Sons, Inc., ref. 345, Copyright © 2007 Elsevier and ref. 346, Copyright © 2009 Elsevier.

Thus far, these dendrimer-based DNA films have mostly been associated with the assembly of DNA chips, sensors and arrays since the components making up their well-defined and organized

layers are essential in such devices. This is of particular importance in reusable devices, where stability under varying conditions, storage and repeat cycles of usage are essential requirements. In the case of interest, readers are referred to the review by Caminade *et al.*³⁴⁸, which is specifically focused on the use of dendrimers in DNA microarrays.

3.3. Layer-by-Layer (LbL) self-assembly

Layer-by-Layer (LbL) self-assembly is a very simple, easy, versatile and inexpensive self-assembly based approach to produce thin multilayered films. The main differences in relation to the previous techniques are that the components of the LbL film do not present an organized structure and that LbL assembly can be applied to planar or colloidal substrates either to coat particles or to produce capsules, respectively^{349–351}.

The pioneers in producing electrostatic interaction-based LbL films having DNA as one of the components was Decher's group^{271,352–358}. Similar to LB films, the most common interactions between the molecules and the substrate making up the LbL film are non-covalent. In fact, adsorption of the multiple layers in LbL films is predominantly driven by electrostatic interactions between oppositely charged entities that are attracted to each other. Because of this, film stability is highly influenced by environmental conditions such as pH, ion concentration, temperature, etc.²⁸⁴. Other driving forces involved in the LbL step-by-step approach that influence the deposition of a layer onto the substrate and that contribute to the stabilization within the film, include metal-ligand coordination, hydrogen bonds, biospecific host-guest interactions and even covalent bonds^{89,276,359}. In the latter case, these interactions would significantly improve the strength and stability of the multilayers.

Briefly, in the LbL self-assembly based approach a given substrate is exposed to an oppositely charged polyelectrolyte solution either by dipping or by addition³⁶⁰. After incubation for a certain amount of time, a first layer of the polyelectrolyte will have adsorbed onto the substrate surface (Figure 47). The formed layer is then washed in order to remove any non-adsorbed molecules and to avoid contamination of the solution in the next step. At this stage, the substrate with the first adsorbed layer is exposed to a second oppositely charged polyelectrolyte solution. This cycle is then repeated until the desired number of layers is achieved, with each deposition step resulting in a reversal of the surface charge.

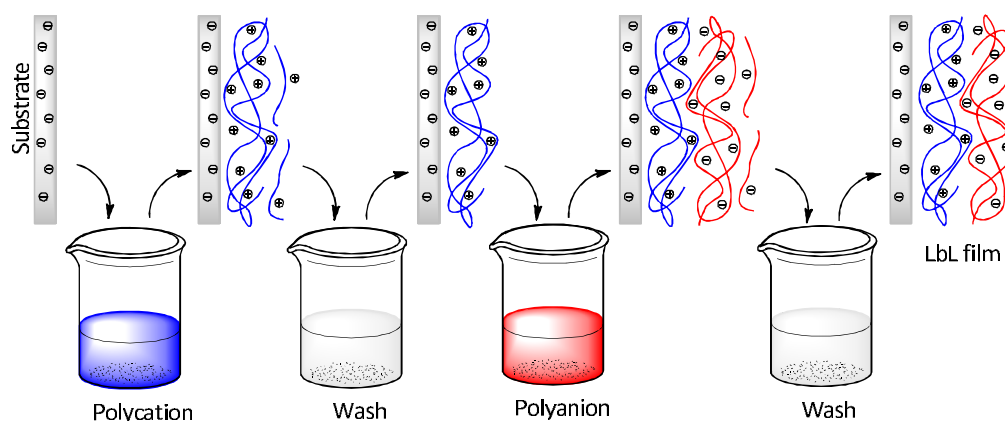


Figure 47 - LbL multilayered film deposition on a planar substrate using the dipping method. The formation of the multilayered film is driven by electrostatic interactions, with charge reversal after exposure to each deposition solution. Adapted from³⁶¹.

In the case of colloidal substrates, the polyelectrolyte solutions are normally added to the substrate particles sequentially, alternating between oppositely charged solutions. Centrifugation steps are used between each polyelectrolyte addition in order to remove the deposition and washing solutions. The incubation time of the substrate in each solution will depend on the type and nature of the layer components and will vary according to the adsorption and saturation kinetics of each component on the substrate surface³⁶².

In general, the LbL self-assembly approach is the most popular liquid phase technique used to obtain multilayered films. Various planar and colloidal substrate types have been modified using this technique to produce LbL films. Some of these substrates however may not have enough charge to support a full multilayer film. In such cases, it is very common to use an initial coat of a different polymer with specific functional groups as film precursor to ensure a well-charged uniform substrate surface³⁶³. Regarding the multilayered films, a wide variety of deposition materials have also been applied onto the layers, namely, nanoparticles and nanowires (e.g. gold, titanium, silica, copper, zinc, graphite and silver)^{364,365}, synthetic polymers and copolymers (e.g. PLL, poly(glutamic acid) (PGA), poly(allylamine hydrochloride) (PAH), PARG, poly(diallyldimethylammonium chloride) (PDDA), PEI, poly(4-styrenesulfonic acid) sodium salt (PSS), poly(vinyl soloxane) (PVS), poly(acrylic acid) (PAA), PAMAM dendrimers, pNIPAM, PEG) and natural polymers (e.g. proteins: albumin, collagen, gelatin, PrS, enzymes; polysaccharides: CHI, ALG, hyaluronic acid (HA), Dx; nucleic acids: DNA and RNA)^{366–371}. LbL films are also known among the scientific community as polyelectrolyte multilayers (PEMs). Biomolecules are well-suited to be submitted and incorporated into LbL films since the deposition method is usually performed in aqueous solutions and in very soft conditions, without any harsh reagents or experimental conditions. Because of this, LbL films have been widely applied in the

biomedical field for, among other applications, cellular adhesion and proliferation, biomaterial coatings, biomimetism, single cell studies and 3D cell architectures³⁷².

LbL assembly is also one of the most common techniques used to produce multilayered DNA films. As DNA is a negatively charged polymer, it has been incorporated into LbL films along with positively charged polymers. This has been achieved exclusively using DNA or previously prepared DNA/polymer complexes (polyplexes)³⁷³. Numerous studies have been reported on the topic of DNA films prepared by the LbL technique^{345,355,356,374–418}. Some of the most interesting works will be briefly discussed next.

Deeply involved in research around LbL films, are the groups of Decher and Caruso. In one of their earliest studies, Lvov, Decher and coworkers prepared ultrathin films by the successive deposition of alternate layers of DNA and PAH onto a PSS/PAH precursor layer on a glass slide³⁵⁶. The thickness of the DNA layer in the film was observed to be bigger than that of the polymer layer. This was mainly attributed to the structural rigidity of the DNA helix and the possible occurrence of some DNA double layer or coiling. The film also presented temperature sensitivity when heating above the T_m of DNA. Still in the same working group, Sukhorukov *et al.*^{355,394} used quartz or silicon substrates pre-coated with PEI to prepare films by first adsorbing DNA with or without a PAH/PSS precursor layer, followed by the alternating adsorption of DNA with positively charged PEI, PLL or PARG.

A study by Caruso and coworkers soon followed for the development of DNA hybridization detection sensors⁴⁰⁵. Here, the synthesis process involved the sequential deposition of alternate layers of DNA and PAH/PSS/avidin, with the latter component playing a role in promoting biotin-DNA adsorption *via* the biospecific interaction between the two molecules^{405,419}. This same immobilization of DNA *via* avidin-biotin biospecific interactions using DNA-based LbL films was also reported by Wang *et al.*³⁶⁸.

Johnston *et al.*³⁸⁰ later explored a very interesting approach for preparing DNA films by the LbL technique, where DNA alone was used (HCR-like process). This was first time that LbL films were prepared using polyelectrolyte with a charge with the same signal. The strategy used involves the preparation of a hydrogen bond-based LbL film that relies on DNA's ability to hybridize with complementary oligonucleotide sequences. Here, instead of alternating oppositely charged polymers, partially complementary DNA strands are added in an alternated manner. This allows for the hybridization between the complementary DNA segments in the alternate layers, with the free ssDNA segments hybridizing to the following DNA strand layer (Figure 48, A). In this case, hydrogen bonds between complementary strands are the driving force for DNA film formation. The film obtained using this approach revealed to be sensitive to low ionic strength solutions due to the phosphate backbone deshielding effect. Deposition of the same DNA-based LbL film onto silica microparticles was also successful (Figure 48, B). Dissolution of the silica template in the films here

resulted in the formation of hollow DNA capsules. Johnston and coworkers also used different DNA sequences to prepare other DNA-based LbL films in order to study film buildup behaviour, viscosity and stability³⁸⁵. The next step involved increasing the film stability under physiological conditions and low ionic strength by incorporating DNA strands as crosslinkers (Figure 48, C). This ensured that the DNA-based LbL film was still biodegradable and biocompatible, since chemically modified DNA or "chemical" crosslinkers were not used³⁹³. Indeed, the crosslinked films prepared here were stable in low salt concentrations (25 mM) when compared with the non-crosslinked films that required a minimum of 200 mM salt concentration to remain stable. A systematic study was performed by Lee *et al.*⁴²⁰ where DNA films were exposed to different salt concentrations (Figure 48, D-E) and different thermal conditions (Figure 48, F-G). The stability of the films was shown to be dependent on the DNA strand length and sequence. When exposed to low salt concentrations, the mass loss in the DNA films was higher. This may possibly be due to the increase in repulsion between the DNA strands, which leads to the disruption of the layers that are then able to rehybridize when introduced back into higher salt concentrations. In the case of the films prepared with longer DNA strands, the mass loss recorded was inferior due to the higher duplex stability. Several other studies have since followed. For example, the investigation of the influence of salt on film assembly⁴²¹, followed by studies in which Förster resonance energy transfer (FRET) was used to monitor film assembly⁴²², modeling the DNA hybridization and conformations formed during film construction^{423,424} and the controlled degradation of the films through the inclusion of restriction enzyme cut sequences⁴²⁵ or competitive hybridization⁴²⁶.

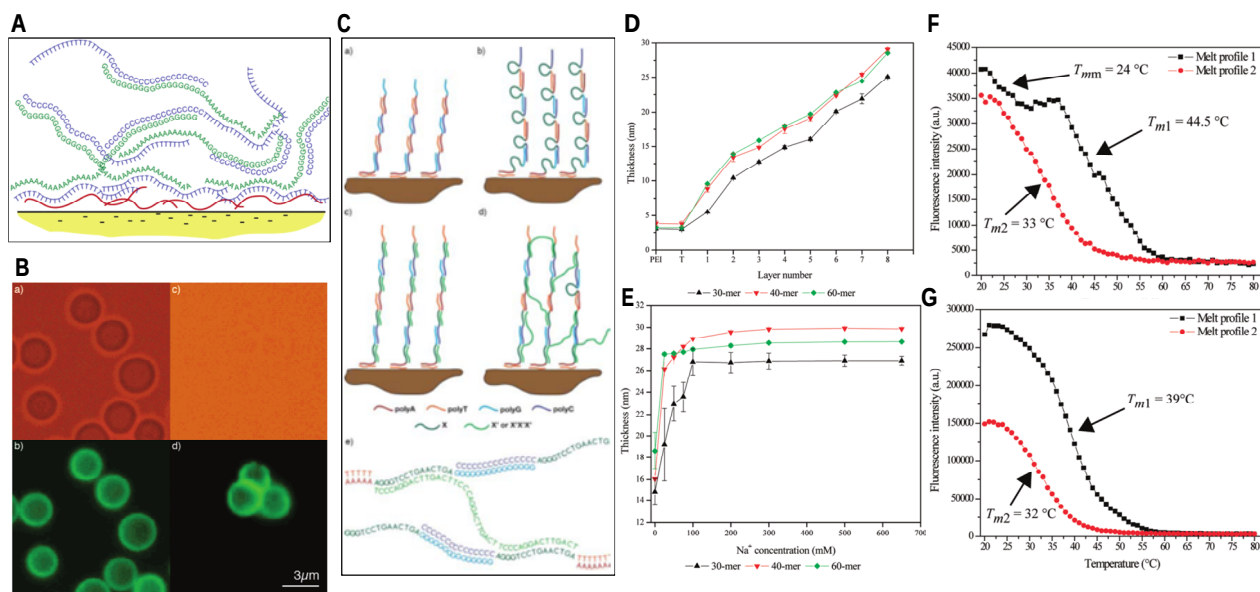


Figure 48 - LbL-based DNA film formation, where solely DNA strands are used for deposition. (A) DNA LbL film deposition on a PEI precursor layer (red lines), using polyA and polyT complementary DNA strands³⁸⁰. (B) 8-layer DNA film-coated silica microparticles in bright-field (a, c) and hollow capsule in fluorescence imaging (b, d)³⁸⁰. (C) DNA film built with different DNA strands (a) diblock and (b-d) triblock. After assembly, the triblock film was hybridized with a X' short strand (c) or crosslinked with a X'X'X' long strand. (e) Crosslinked DNA film scheme in detail³⁹³. (D) Influence of DNA strand on the thickness of the film (odd and even layers correspond to the 30-mer A₁₅G₁₅ and T₁₅C₁₅ DNA strands, respectively)⁴²⁰. (E) Thickness of different length DNA strand 4-bilayer films in different salt concentrations⁴²⁰. (F) Melting profile for 3.5 bilayer film terminating in A₁₅G₁₅, and (G) for 4 bilayer film terminating in T₁₅C₁₅, in each case with T_{m1} (melting temperature of the film) and T_{m2} (melting temperature of the rehybridized strands in solution)⁴²⁰. Adapted/reprinted with permission from ref. 380, Copyright © 2005 American Chemical Society, ref. 393, Copyright © 2008 by John Wiley & Sons, Inc., and ref. 420, Copyright © 2008 American Chemical Society.

These works combined clearly point to the potential of DNA-based LbL films as candidates for several applications, such as gene and/or drug delivery systems that display controlled and sustained release, targeted delivery, diagnostics and microreactors. In another study, Lazerges *et al.*³⁹⁶ constructed LbL DNA films using complementary DNA strands but starting with a SAM of thiol-modified DNA as the film precursor layer. The prepared system was also considered to be of interest for potential application in biosensors and bioelectronics.

In a recently published work by Shim *et al.*⁴¹³, a very out-of-the-box strategy using DNA-driven LbL assembly was developed. By alternating monolayers of ss-DNA grafted AuNPs (GNPs according to the authors) (Figure 49), thin self-standing films consisting of an active and a passive layer were acquired. In the presence of the corresponding complementary ssDNA strands, the prepared films were observed to display a reversible curling behaviour, with each layer responding in the corresponding active or passive manner. For a better understanding of the mechanisms involved in this process, consultation of the published paper is recommended.

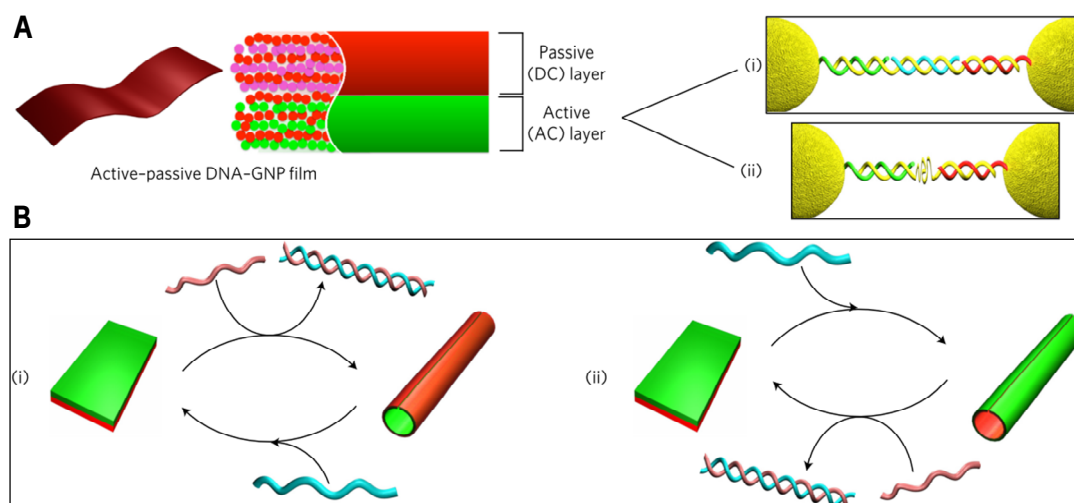


Figure 49 - Active-passive DNA-GNP films. (A) Films composed of GNPs functionalised using 3 different ssDNA molecules, namely, A, C and D. The ssDNA combination AC is the active layer (green) and the DC combination is the passive layer (red). On the other hand, the AC layer could be built with either a ssDNA segment with filler (i) or a ssDNA segment without filler (ii). (B) Programmed actuation of the DNA-GNP films, where flat films were induced by the addition of either (i) a stripper AC strand or (ii) a filler AC strand to form tubular structures where the (i) passive or (ii) active layers acted as an outer layer by (i) contraction or (ii) expansion of the active layer, respectively. ⁴¹³ Adapted/reprinted with permission from ref. 413, Copyright © 2016 Springer Nature.

David M. Lynn's research group has also embraced the LbL assembly approach to develop DNA films. The main goal of their work was focused on the use of LbL DNA films in gene delivery and cell transfection studies, envisioning final *in vivo* application of the films in the medical field. Typically, 8 bilayers of synthetic degradable polyamine (*i.e.* polymer 1) were alternately deposited with pDNA encoding enhanced green fluorescent protein (pEGFP) onto quartz and silicon planar substrates, each pre-coated with 10 bilayers of linear PEI/PSS (PSS is SPS according to the authors)³⁸¹. It was concluded that the obtained polymer 1/pDNA film was 100 nm in thickness and that its deposition onto the coated substrates was concentration, ionic strength and pH-dependent (Figure 50, A-B)³⁷⁸. Film degradation was also observed to occur hydrolytically, in a controlled and sustained manner under physiological conditions (Figure 50, C). This encouraged the release of pDNA from the film into solution, its subsequent uptake by cells and finally cell transfection (Figure 50 Figure 50, D). An interesting approach to locally promote and detect cell transfection was to place the quartz slide that was coated with the DNA film directly on top of the cells (Figure 50, E-F)³⁸².

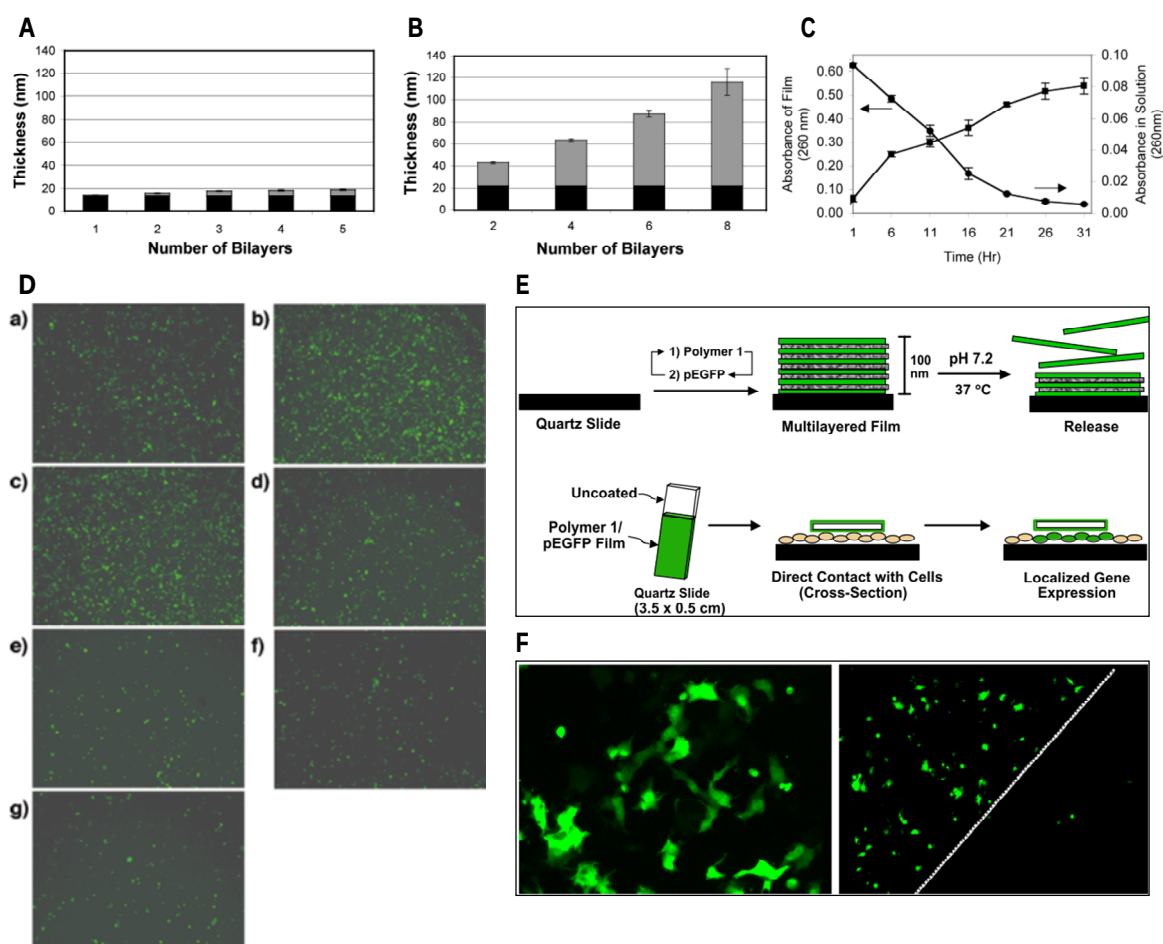


Figure 50 - Degradable polymer 1/pDNA LbL film. Variation of thickness as a function of number of polymer 1/pDNA bilayers, where pDNA was prepared in (A) deionized water and (B) in 100 mM sodium acetate buffer (pH = 5.1). In each case, the black bar corresponds to precursor LPEI/PSS layer³⁷⁸. (C) Erosion assay where the absorbance at 260 nm for DNA in the film and DNA released into solution was measured over time, with film incubation occurring under physiological condition (PBS, 37°C)³⁷⁸. (D) Fluorescence microscopy images showing cells transfected with DNA from the release solutions obtained at different time points in the erosion assay, where Lipofectamine 2000 was used as transfection reagent. Time points a) 0-1h, b) 1-6h, c) 6-11h, d) 11-16h, e) 16-21h, f) 21-26h, and g) 26-31h³⁷⁸. (E) LbL DNA film deposition and release under physiological conditions (top) and direct contact of film with cultured cells, promoting cell transfection and gene expression³⁸². (F) Fluorescence microscopy images of cell expressing EGFP 48 h post-contact³⁸². Adapted/reprinted with permission from ref. 378, Copyright © 2004 American Chemical Society and ref. 382, Copyright © 2005 Elsevier.

As a next step, Lynn and co-workers broadened their systematic studies to include DNA-based LbL film preparation on other substrate types. For example, DNA films deposited onto stainless steel intravascular stents³⁸⁹ were assessed in terms of their stability and integrity when exposed to the mechanical challenges that may be encountered in a surgery scenario. Here, the coated stents were capable of mediating cell transfection as was evidenced using either Lipofectamine (commercial transfection reagent) or a direct contact procedure. Implementation of the LbL approach in this kind of medical apparatus takes advantage of several factors including organic solvent-free conditions, reduced amount of materials used and sustained DNA release. Scale-down is another factor to

consider when preparing materials for such purposes, particularly when the starting material is generally at the macroscale. Following this rationale, DNA-based LbL films were also deposited onto polystyrene (PS) microspheres³⁹⁸ and stainless steel microneedles⁴⁰³ for application in cell transfection/macrophage internalization and transdermal drug delivery, respectively. Another interesting study also developed by this research group, demonstrated that DNA film erosion was driven by hydrolytic cleavage of the charge-shifting polymer with which the LbL film was built³⁹⁵. As a result of cleavage, carboxylic groups were introduced into the polymer chain, thus reducing the net charge of the polymer and decreasing the electrostatic interaction with DNA.

Still in the gene delivery field, the group of Jian Ji have been working with biodegradable DNA films^{379,383,384,386,397,408}. Ren *et al.*³⁷⁹ for example evaluated the effect of ionic strength on the construction and deconstruction of a 10 bilayer PLL/DNA film (Figure 51, A). It was shown that 0.5 M NaCl was the optimal salt concentration for film growth to occur, with the ions playing an important role in maintaining DNA conformation. At low salt concentrations, the DNA molecules were likely stretched due to the negative charges of the phosphate groups in the DNA chain repelling each other. With increasing salt concentrations, the presence of salt ions shielded the chains possibly leading to the DNA molecules coiling up or intertwining together. At very high salt concentrations however the shielding effect was too high, disabling the electrostatic interaction between DNA and the polymer (Figure 51, B-D). The same effect was reported for erosion of the films (Figure 51, E-F). The PLL/DNA films here were also biodegradable *via* enzymatic degradation by α -chymotrypsin³⁸⁶. Even a controlled and sustained release was achieved (Figure 51, G) by preparing the same film followed by chemical crosslinking it with GA³⁸⁴.

In terms of their use for gene delivery applications, DNA-based LbL films were built by alternately depositing a polymer layer with a layer of polyplexes made up of DNA and another polymer³⁹⁷. Briefly, prior to film deposition, DNA was complexed with CHI. These polyplexes were then incorporated into a multilayer assembly *via* the LbL approach with the oppositely charged HA, with GA being used as a crosslinker. This approach, contrary to Lynn's work, takes advantage of the DNA polyplexes incorporated into the LbL film that, upon their release, were directly taken up by the cells allowing for transfection. In the non-viral gene delivery system developed here, the formed polyplexes incorporated into the LbL film still conferred DNA condensation, surface charge, protection against enzymatic degradation and cellular internalization⁴²⁷ (Figure 51, H). At the same time, the use of natural polymers such as CHI and HA to encase the polyplexes ensured biocompatibility (Figure 51, I). Thus, the benefits of using this DNA-based LbL film system for potential application into medical devices and tissue engineering scaffolds are great.

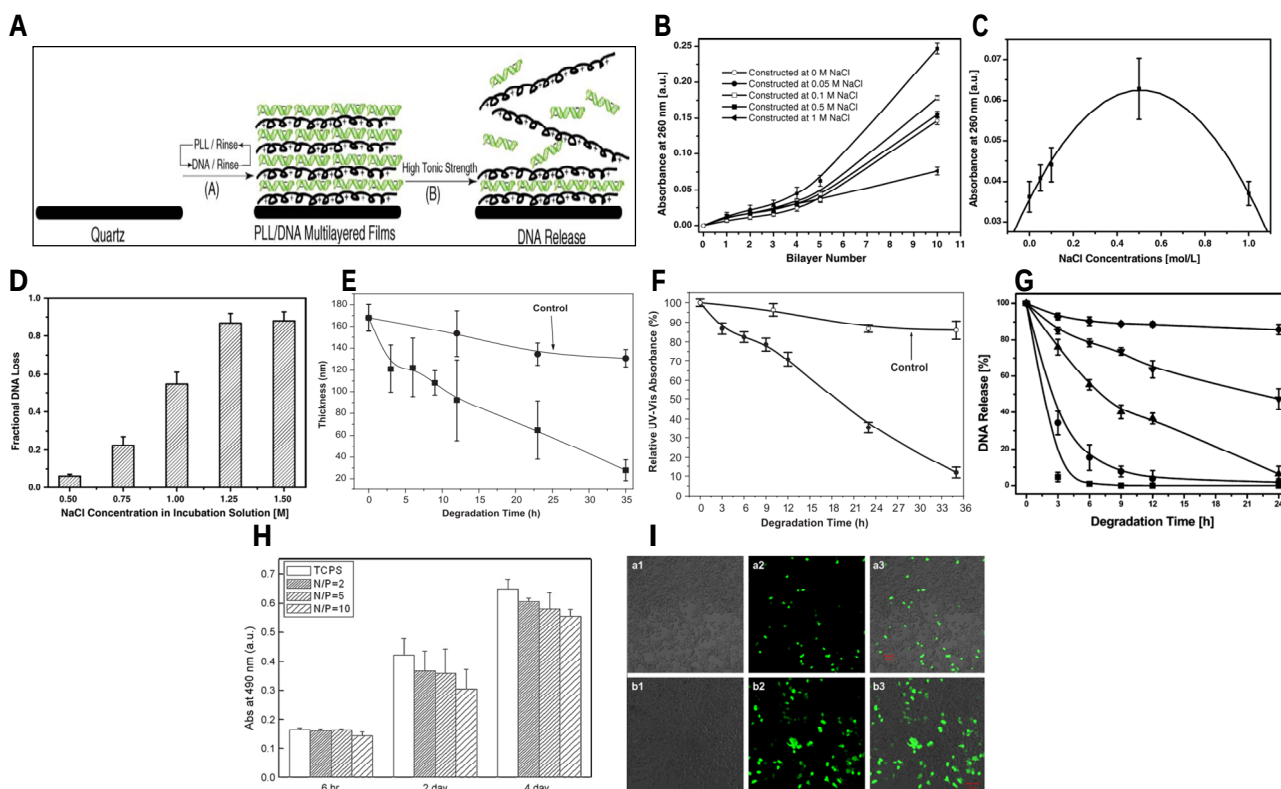


Figure 51 - Multilayered DNA-based LbL films for gene delivery. (A) Construction and salt-induced deconstruction of PLL/LbL DNA films³⁷⁹. (B) DNA UV-vis absorbance of the PLL/DNA films as function of layer assembly at different salt concentration³⁷⁹. (C) DNA UV-vis absorbance of the PLL/DNA films as function of salt concentration³⁷⁹. (D) DNA loss after 12h incubation in solutions with different salt concentrations at 37°C³⁷⁹. (E) Film thickness variation with enzymatic degradation time³⁸⁶. (F) Relative UV-vis absorbance of DNA film as a function of degradation time³⁸⁶. (G) DNA release from PLL/DNA films when using a trypsin solution at 37°C with varying crosslinking time (square: non-crosslinked film; circle: 10 min; triangle: 30 min; inverted triangle: 60 min; diamond: 180 min)³⁸⁴. (H) Cell viability when using multilayered films built with HA and CHI-DNA complex³⁹⁷. (I) EGFP gene expression in cells cultured on (a) 1 bilayer and (b) 5 bilayers of HA/CHI-DNA complex³⁹⁷. Adapted/reprinted with permission from ref. 379, Copyright © 2005 Elsevier, ref. 384, Copyright © 2006 American Chemical Society, ref. 386, Copyright © 2005 Elsevier and ref. 397, Copyright © 2009 Elsevier.

Dendritic polymers have also been used to assemble DNA-based LbL films either on planar or in colloidal substrates for DNA biosensor and drug/gene delivery applications. Yu *et al.*³⁹⁹ used positively and negatively charged phosphorous dendrimers to assemble a LbL film on a gold-coated glass substrate. This was followed by the covalent immobilization of ssDNA onto the film, which was then used for the detection of DNA hybridization. In another study, Kim *et al.*⁴²⁸ assembled a dendrimer/DNA multilayered film onto microparticles, which after template dissolution led to the formation of hollow microcapsules. Incorporation of dendrimers into DNA LbL films *via* electrostatic interactions has also been performed where the dendrimer/DNA complexes were first prepared and then incorporated into the multilayered film by the LbL technique^{429,430}. In other studies, Liu and coworkers constructed water-insoluble LbL DNA films and analyzed the interaction between the DNA in the prepared films with intercalating dyes such as EtBr, AO, 9-aminoacridine hydrochloride (Aa)

and 5,10,15,20-tetrakis(4-N-methylpyridyl)porphine tetra(p-toluenesulfonate) (TMPyP)^{376,377,418,431}. Through these works, it was possible to determine whether the interactions established between DNA and the different dyes occurred as a result of intercalation or electrostatic binding. Factors including sequence dependency, saturation and the stability of the DNA-dye complex under different environmental conditions such as temperature and pH were also evaluated here.

The reductive nature of the intracellular environment has been shown to be a trigger to promote LbL DNA film disassembly allowing for DNA and drug release applications, by Ouppický's group. For example, the inclusion of labile disulfide bond-containing polymers in the construction of LbL DNA films was achieved by Blacklock *et al.*^{391,404,407} and by Zou *et al.*⁴¹⁰ when they incorporated the TAT-polypeptide (Figure 52, A) or reducible hyperbranched poly(aminoamine)(PAA) polymers into the films. In the presence of reductive molecules such as 1,4-Dithiothreitol (DTT) or GSH, the disulfide bonds in the polymers were disrupted (Figure 52, B-C) and the stability of the films was compromised, thus allowing for the controlled release of DNA during the cell transfection process. Similarly, Hujaya *et al.*^{411,412} used the same bio-reducible PAA polymers in their strategy to produce thin multilayered DNA films. The PAA polymers were modified with cystamine bisacrylamide (CBA) combined with 4-aminobutanol (ABOL), histamine (HIS) or 1,4-diaminobutane (DAB) leading to the formation of p(CBA-ABOL), p(CBA-HIS) and p(CBA-DAB), respectively (Figure 52, D-F). These modified PAA were then deposited in silicon substrate, along with calf thymus DNA or a pDNA expressing enhanced green fluorescent protein (GFP), the latter one to access cell-transfection capability of the modified surface. The release of pDNA, for posterior cell transfection, was possible due to the labile bonds present in the polymer, that broken in a reductive environment.

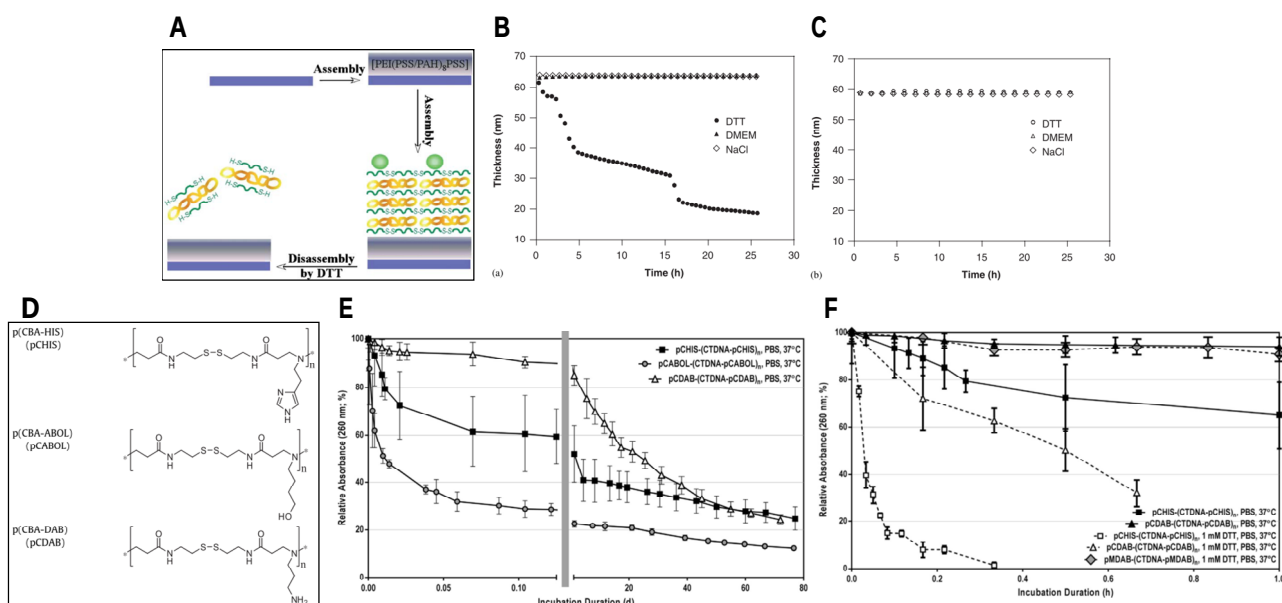


Figure 52 - DNA films with reducible disulfide bonds. (A) Assembly and disassembly of reductively degradable TAT/DNA films³⁹¹. (B) TAT/DNA film thickness variation in the presence of DTT, DMEM cell culture medium and NaCl solution³⁹¹. (C) Control PLL/DNA film without disulfide bonds and the analysis of thickness variations in the presence of the same solutions³⁹¹. (D) Chemical structure of bio-reducible PAA polymer possessing a S-S bond⁴¹¹. (E) DNA release of PAA/DNA multilayered films under physiological conditions, with graph on the left showing a burst release within 3h and graph on the right showing a sustained release⁴¹¹. (F) DNA release profiles of pCHIS-(CTDNA-pCHIS)₁₀ and pCDAB-(CTDNA-pCDAB)₁₀ with and without DTT⁴¹¹. Adapted/reprinted with permission from ref. 391, Copyright © 2006 Elsevier and ref. 411, Copyright © 2015 Elsevier.

The fabrication of LbL DNA films based on metal-ligand coordination have also been reported by Xu *et al.*⁴³² and Wang *et al.*⁴³³, in which the LbL assembly of the multilayered film was driven by metal-ligand coordination/electrostatic forces. In the first study (Figure 53, A), the construction of multilayered aluminum(III) alkanebisphosphonate (Al₂(C₄BP)) allowed for the immobilization and hybridization of ssDNA strands, which was detectable by electrogenerated chemiluminescence after oxidation of Ru(bpy)₃²⁺ linked to the DNA⁴³². In the second study which envisaged cell transfection, a SAM modified gold substrate was alternately dipped into inorganic zirconium ion (Zr⁴⁺) and DNA solutions⁴³³. The coordination and electrostatic interactions between the two components resulted in a LbL DNA film (Figure 53, B) with an electrodisassembly behaviour. That is, when the Zr⁴⁺/DNA films were subjected to an electric potential, they were observed to disassemble due to the cathodic electrolytic deposition of zirconia (Zr⁴⁺ reduced to Zr). Consequently, DNA was released into the medium and the cells were transfected. The most exciting observation regarding this disassembly approach was the dramatic reduction in biocytotoxicity since the ions were reduced and deposited onto the cathode.

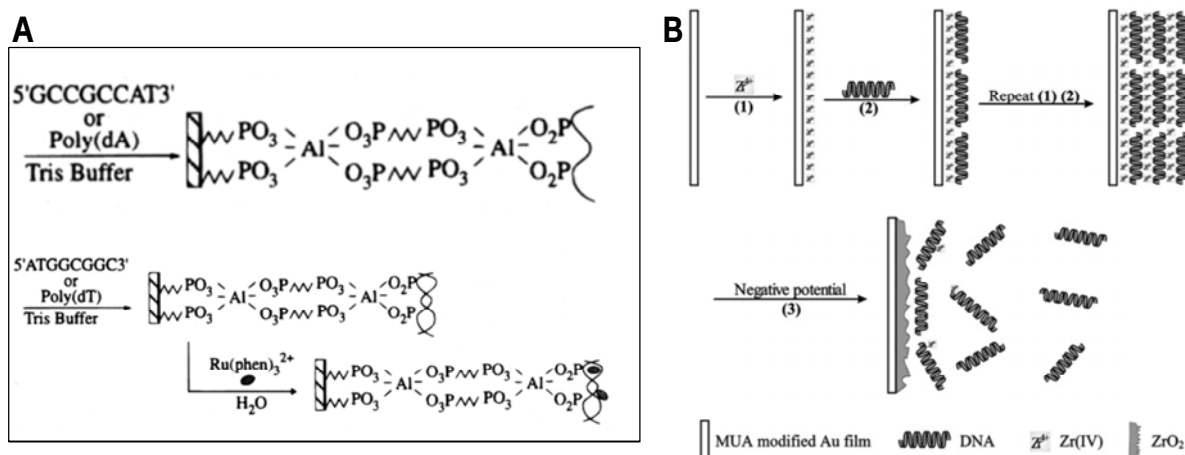


Figure 53 - LbL DNA films stabilized by metal-ligand coordination. (A) Sequential deposition of $\text{Al}_2(\text{C}_4\text{BP})$ film on gold surface followed by DNA immobilization, hybridization and Ru(phen)_3^{2+} interaction with dsDNA⁴³². (B) Zr^{4+} and DNA LbL assembly, subsequent electrodisassembly of the multilayered film and release of the DNA⁴³³. Adapted/reprinted with permission from ref. 432, Copyright © 1995 American Chemical Society and ref. 433, Copyright © 2009 The Royal Society of Chemistry.

Many other interesting studies exist based on electrostatic interaction driven-LbL DNA films that are used as sensors, biosensors, chips and biomedical devices to either electrochemically detect DNA damage⁴¹⁴, monitor film assembly and adsorption kinetics^{415–417}, achieve surface-mediated gene and drug delivery with a controlled and sustained release under physiological conditions^{373,374,390,409,434}, access DNA binding drugs and dyes^{388,401}, potentially screen for drugs, immunoassays, genotyping and gene expression profiling³⁸⁷, transdermal drug and vaccine delivery⁴⁰⁰, and improve surface bioactivity of biomedical materials and devices⁴⁰⁶.

Summarizing, the assembly of the DNA-based LbL films discussed up to this point were mostly based on the electrostatic interactions between oppositely charged polyelectrolytes, irrespective of posterior crosslinking using chemical (e.g. GA) or physical (e.g. UV) methods. Also, few examples about biospecific interactions, hydrogen bonding and metal-ligand coordination-based LbL DNA films have been addressed³⁰⁴.

3.4. Solution cast deposition and wet spinning methods

The previously discussed techniques used to obtain ultrathin and thin DNA films have been widely implemented in the materials science community. In the case of thicker and bulky DNA films, which have also been produced and used in scientific research purposes, other solution deposition techniques have been applied to obtain pure or hybrid DNA, the most common being drop or

solution casting (Figure 54). Spin-on coating is considered a variation of the cast method (spin casting). Combined, all these techniques are very simple and straightforward.

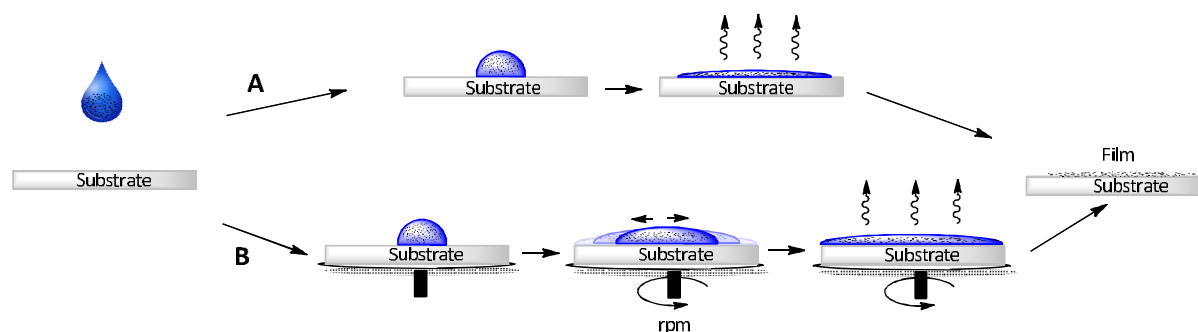


Figure 54 - Solution casting deposition techniques. (A) Drop casting: Solution with the film components is deposited and uniformly spread on the substrate, followed by either draining or evaporation of the solvent. (B) Spin casting: The solution is deposited onto the substrate and it is then spread by applying centrifugal force, followed by solvent evaporation.

The deposition of a solution (made up either a polymer or a mixture of components) onto a substrate, followed by its even spread and solvent removal, leaving behind a dry layer polymers on the substrate surface, results in a cast film. This is a technique that has also been applied to obtain DNA films.

The first researchers to produce a cast film incorporating DNA in its composition were Daoust and Amano, with a very interesting work in which the DNA cast films were used to detect and localize the enzymatic activity of DNase in tissue sections⁴³⁵. Thereafter, and as of 1993, the group of Okahata has been most involved with research focused on DNA cast films. In their approach, DNA is first mixed with a cationic lipid, resulting in a precipitate that is then centrifuged and dried⁴³⁶. The particularity of the DNA-lipid complex is that it is soluble in hydrophobic organic solvents but not in aqueous solutions. Deposition of this cast film onto a quartz-crystal microbalance (QCM) crystal, where it maintained its water-insoluble self-standing properties (Figure 55, A-B), was seen as an advantage to detect and study the intercalation of dyes such as EtBr with DNA without the risk of film dissolution. Also, it was found that by varying the water content in the organic solvent solution, the DNA helical conformation in the films interchanged between the B and Z-forms and were aligned when the films were stretched in one direction⁴³⁷. Due to the energy transfer and conduction of π electrons along the DNA strands and the ability to produce oriented DNA-lipid complexes within the films (Figure 55, C), electric conductivity through the DNA cast films could be measured^{438,439}. On unstretched films where DNA strands were unoriented, a small current was detected but this was not reproducible. In contrast, two scenarios were presented for the stretched DNA cast films (Figure 55, D). If a film was placed with the dsDNA strands aligned parallel to the two electrodes, the electric current was low. Conversely, when the dsDNA strands were perpendicular to the electrodes, a large

current was observed. The nano-organization of a DNA-lipid/dye complex was also studied⁴⁴⁰. A comparison of the electric conductivity of DNA cast films and the previously mentioned LB films was also made²⁹⁶. Nakayama *et al.*⁴⁴¹ found that the intramolecular electron conductivity of a DNA-aligned cast film was dependent on DNA strand length as well as on temperature. Films prepared with small DNA strands did not present significant currents, whereas films made up of long DNA strands were able to bridge the distance between the electrodes allowing for constant and large currents. In the latter case, electron conductance was reversible with temperature variations, with high temperatures (always below the T_m of DNA) resulting in lower conductance, possibly due to DNA premelting effects⁴⁴².

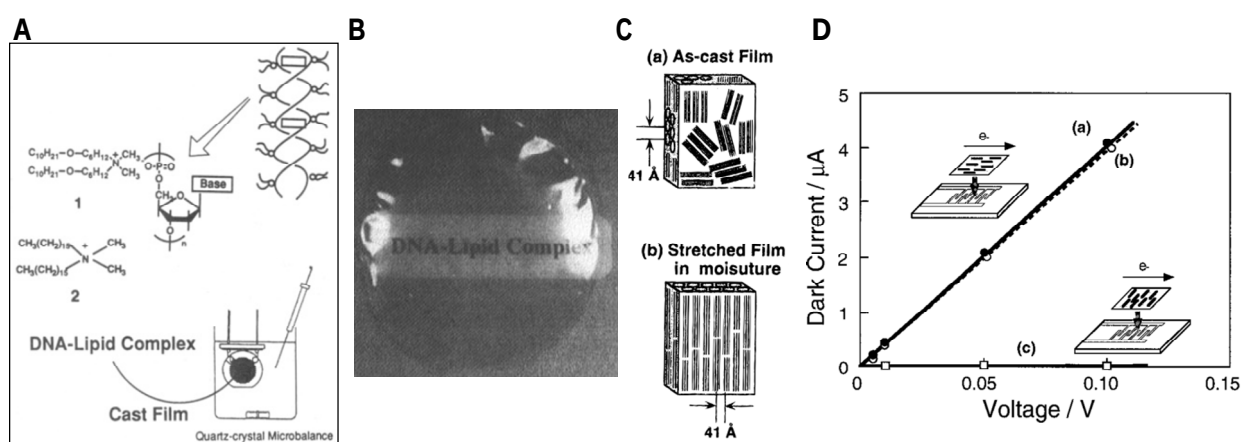


Figure 55 - DNA-lipid cast films. (A) Quartz-crystal microbalance (QCM) coated with a DNA-lipid complex cast film⁴³⁶. (B) Photograph of a self-standing DNA cast film⁴³⁷. (C) (a) Unstretched, not oriented and (b) stretched, oriented DNA cast films with the DNA strands represented as black lines⁴³⁷. (D) Dark current of aligned DNA films with (a) perpendicular and (b) parallel positioning in the electrodes⁴³⁹. Adapted/reprinted with permission from ref. 436, Copyright © 1993 American Chemical Society, ref. 437, Copyright © 1996 American Chemical Society and ref. 439, Copyright © 1998 Elsevier.

Later in 2008, the research group of Venkatraman fabricated DNA cast films. Their strategy was to encapsulate either naked or complexed DNA into polymer films for posterior use in sustained release and cell transfection studies. Ramgopal *et al.*⁴⁴³, for example, prepared complexes made up of pDNA expressing EGFP and the commercially available DNA delivery system, Lipofectamine. The formed lipoplexes were then mixed with either gelatin/polycaprolactone (PCL, a degradable polymer) or gelatin/poly(ethylene-co-vinyl acetate) (PEVAC, a non-degradable polymer) and were finally cast as films 80 μm in thickness. With the naked pDNA/PCL films, an initial burst release of pDNA was observed due to the low solubility of PCL. In the pDNA/PCL/gelatin films, on the other hand, the burst effect was suppressed as the pDNA had partitioned into the gelatin phase of the film. In the cast films incorporating PEVAC, the pDNA burst release occurred at the same rate with or without gelatin. Overall, the release of naked pDNA was higher in the PCL-based films relative to the PEVAC-based

films owing to the degradability of PCL. In the case of the films prepared with the pDNA-Lipofectamine lipoplexes, no initial burst release was observed and pDNA release was found to occur slowly with gelatin playing a role in enhancing the release. Combined, these factors may compromise the therapeutic goal of the prepared DNA cast films. Regarding the transfection studies, the prepared lipoplexes in general were found to be more efficient. Posteriorly, a comparison was made between PEI/pDNA polyplexes and Lipofectamine/pDNA lipoplexes in PCL/gelatin films⁴⁴⁴. Here, the prepared lipoplexes showed no burst release. However, the presence of gelatin in the lipoplexes was found to enhance the pDNA burst release and also contribute to the successful transfection of cells. In the case of the polyplexes, the release of pDNA was driven by electrostatic interactions and hydrogen bonds between PCL and PEI, while gelatin was found to hinder its release. Further characterization and in-depth studies of the cell transfection and intracellular localization of the lipoplexes were carried on by Mondal *et al.*^{445,446}.

The increasing concerns related to environmental pollution and the hazards associated with DNA intercalating molecules have driven Nishi's group to fabricate pure DNA films as filters to remove such contaminants. To achieve this, the research group focused on casting films either made by mixing DNA with ALG followed by precipitation⁴⁴⁷ or by creating a tridimensional network purely composed of DNA where a dried DNA cast film was exposed to UV radiation to promote crosslinking^{448–450}. The UV-irradiated DNA film (Figure 56) had self-standing properties and was also found to be water-insoluble up to 100°C, where film disassembly was finally promoted by the denaturation of DNA. Moreover, the film was found to be capable of removing and accumulating endocrine disruptors⁴⁴⁹, as well as intercalating molecules such as EtBr and AO that could be easily detected by the resulting color change^{448,450}. Obtaining a water-insoluble DNA film was only possible when dsDNA was used in the preparation process, followed by film drying. Matsumoto *et al.*⁴⁵¹ also used the same strategy for the electrochemical detection and removal of environmental pollutants.

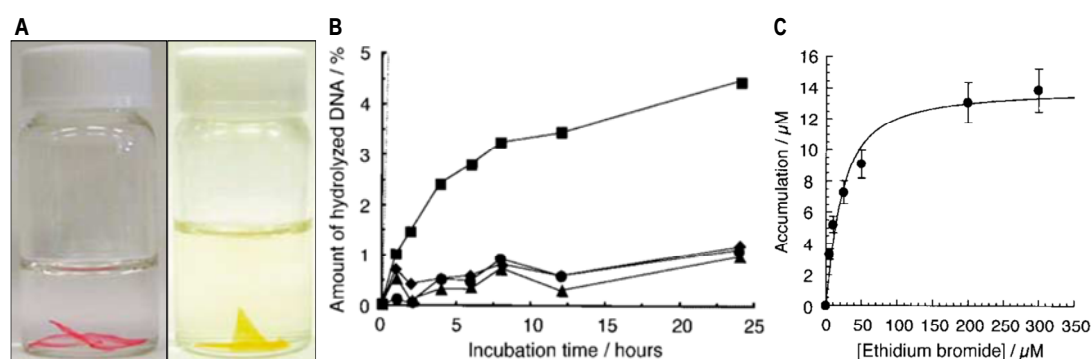


Figure 56 - UV-irradiated DNA film. (A) Accumulation of EtBr (pink) and AO (yellow) by the DNA film. (B) Effect of nuclease at different time intervals on UV-irradiated DNA films (square: 2h and circle: 6h, with nuclease) and (diamond: 2h and triangle: 6h, without nuclease). (C) Binding of EtBr to UV-irradiated DNA immobilized on glass beads.⁴⁵⁰ Adapted/reprinted with permission from ref. 450, Copyright © 2002 by John Wiley & Sons, Inc.

As can be seen by the previous examples, DNA cast films can be easily prepared by deposition and solvent evaporation. They can be made entirely of DNA^{452–456} or in combination with molecules such as lipids, hemoglobin⁴⁵⁷, metallic compounds^{458–460}, ionic liquids^{461–463}, surfactants^{464–466}, diaminepropane⁴⁶⁷, DOX⁴⁶⁸, riboflavin⁴⁶⁹, poly(ethylene oxide) (PEO)⁴⁷⁰ and HRP⁴⁷¹. Generally, these films have been shown to be good candidates in a wide range of applications including biosensors, bioelectrocatalysis, conductive, molecular, optical and optoelectronic devices, radiation degradation studies of DNA, pollutant detection, chemoradiation therapy, DNA-drug interactions and fibre laser applications.

Spin casting is very similar to drop casting, with the biggest difference being the way that the sample is spread on the substrate. Firstly, the solution is dropped onto the substrate. Then the substrate is rotated at a specific velocity (rotations per minute, rpm). The centrifugal force to which the liquid is submitted leads to the drop flattening or spreading outwards from the starting point on the substrate into a planar film⁴⁷². After solvent evaporation, a very thin and uniform film is obtained. Film formation and thickness will depend on several factors including the speed, time, solution hydrodynamics and solvent evaporation⁴⁷³. Very few works with DNA films prepared using this technique have been published^{474–476}.

Wet spinning, on the other hand, relies on the precipitation of the film components into a fibre form. This can be done in the presence of a solvent or in a solution in which the components are not soluble (Figure 57).

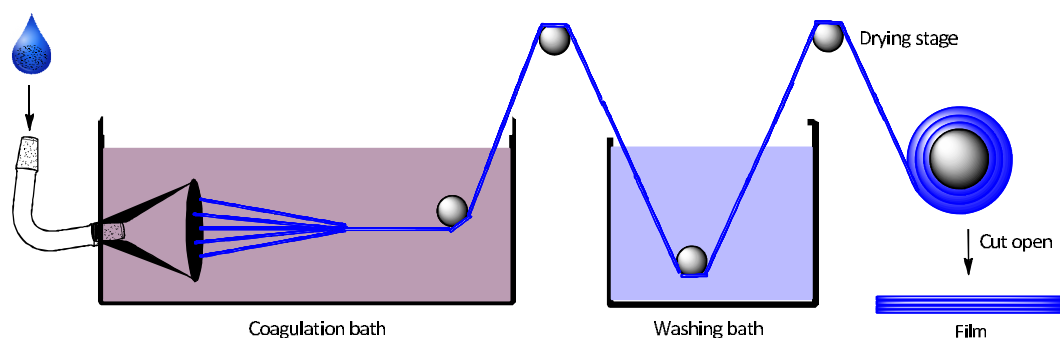


Figure 57 - Simplified scheme of the wet spinning technique. The polymer solution is spun through a spinneret into a coagulation bath to form the fibre, which is then guided through rolling pins to a washing step and finally to a collecting rolling pin. Afterwards, during the drying stage, the oriented fibres fuse together on the pin, forming an oriented film.

Allan Rupprecht^{477,478} developed the method of preparing oriented DNA films using wet spinning in 1966. Firstly, the DNA solution was spun through a spinneret that was already immersed in a coagulation/precipitation aqueous solution made up of 70-85% ethanol and salts. As the DNA solution was spun into the coagulation bath, it immediately coagulated to form a fibre that was then

stretched to orient the DNA strands. The stretched, oriented DNA strands produced were then guided through rolling pins for washing, drying and then finally storage. The successive layering of the DNA fibres on the rolling pin and the subsequent drying process makes them fuse and results in the formation of oriented films. Studies have since proceeded where DNA and DNA complexes (e.g. with AO, proflavine) have been used to prepare films with different physical, chemical, mechanical and biochemical properties. Studies focused on the effects of hydration^{479–483}, DNA conformation and ions^{484–488}, swelling^{489,490}, conductivity⁴⁹¹, optics^{492,493}, effects of radiation^{494–497}, have been conducted using films prepared in this manner. Other researchers such as Kistner *et al.*⁴⁹⁸ and André *et al.*⁴⁹⁹ have also used the same wet-spun DNA films based on Rupprecht's method for their work.

3.5. Applications of DNA-based films

DNA-based films may be suitable platforms for a series of applications, most of them already mentioned along the previous sections. For starting, they undoubtedly constitute an important mean to study the physical and chemical properties of DNA, including those involved in the interaction between DNA and other molecules and ions. Indeed, DNA-based films have been studied regarding DNA's conformational changes upon different environmental stimuli^{484–488}, hydration^{479–483}, swelling^{489,490}, conductivity⁴⁹¹ and degradation^{494–497}, among other parameters. Not only from the point of view of their applicability in solving real problems, but also having in consideration the need to increase the knowledge about DNA itself, DNA-based films studies are providing scientists with extremely precious information. Furthermore, the versatility associated with the methods that may be used in the preparation of the films results in film's structures very different from each other, opening the door to their application in many fields of science and technology. Next, the most important applications of DNA-based films will be briefly summarized.

DNA-based films as components in biosensors

The assembly of highly organized DNA-based films over a surface having free ssDNA exposed for interaction with target molecules has been a popular choice in the biosensor's field. In this kind of devices, researchers search for films having a high stability, and well defined and organized structures. Moreover, when incorporated in a biosensor, the ability of the film to enter a detection-recovery-reuse cycle, without losing its properties and efficiency is very important. These requirements can be achieved mostly with films obtained through the LB technique and surface covalent functionalisation, including SAMs formation. Indeed, the use of biosensors based on ssDNA

or dsDNA prepared using these techniques has allowed the detection of ssDNA (by hybridization)^{294,314,315,321,342}, to the point of distinguishing fullmatch from mismatch hybridization^{316–320}. The process itself has been studied in term of kinetics and stability, allowing the development of not only very sensitive, but also reliable sensor devices^{340,341}. DNA-based films have, thus, important applications in DNA microarrays, for example for the detection of gene expression, and in the detection of genetic mutations.

Nevertheless, DNA does not interact or bind only with other DNA molecules. The interaction and binding of DNA with other chemical entities, such as molecules (like DNA intercalators), ions, ligands, proteins, enzymes, etc., has also been studied^{294,296–303}. DNA-based films can thus be also applied in other types of sensors for specific/targeted detection³⁴⁶. In addition, these devices can be used in studies related with ligand/receptor affinity, *in situ* enzymatic reactions, protein affinity and size exclusion, and electrical properties upon binding to the target^{294,296,330,331,297–301,327–329}. The extent of DNA/target binding has been quantified by diverse modes, for example through electrochemical, charge transfer, mass change and fluorescence detection.

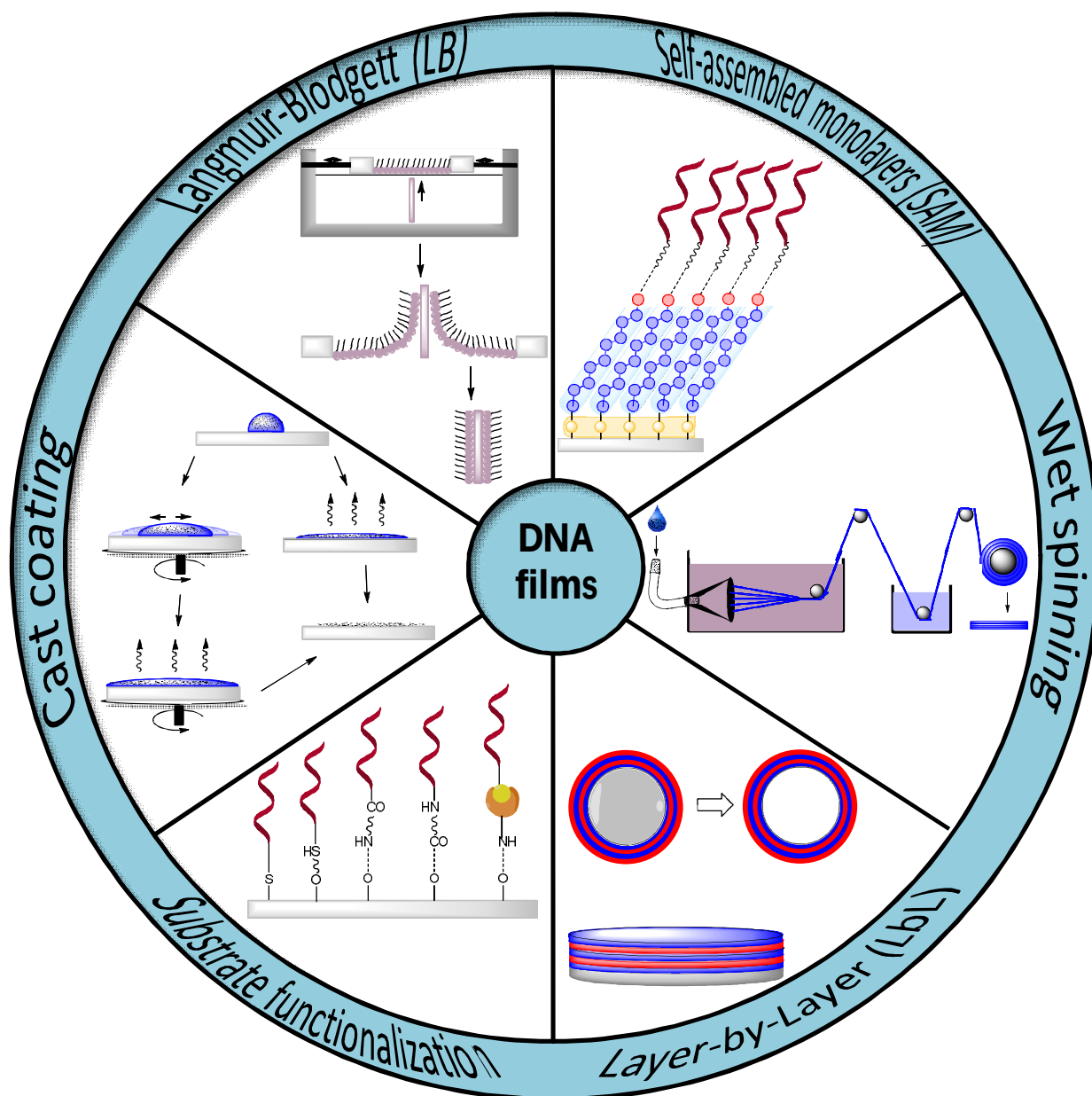
DNA-based films in drug and gene delivery applications

The biocompatibility and biodegradability of the DNA molecule allows the use of DNA-based films in direct contact with the cells in an *in vitro* and *in vivo* scenario. In this scope, among other biomedical applications, gene and drug delivery are particularly relevant. Here, DNA-based films are being studied as components in platforms for the delivery of therapeutic molecules. The films prepared through the LbL technique may be very important in this context as this is a very simple, cheap and easy methodology to carry out. It has also the advantage of being able to be used in water (or other biological fluids) and be applied on substrates of various shapes and sizes. As mentioned before, hollow capsules at the nano and microscale have been prepared using DNA as a building block for multilayer construction⁴²⁸. When the DNA used codifies a gene of interest, this DNA can be transferred to the interior of cells and, simultaneously, to carry drugs in their interior – that is, be used as delivery vehicles for simultaneous drug and gene delivery (gene/drug co-delivery)^{373,374,378,379,381–384,390,391,397,403,404,407–412,427,434}. In these cases, the biodegradability of the films is an important requirement and controlled release is aimed.

DNA-based films can also be prepared using the LbL technique over macroscopic substrates like scaffolds for cell growth and differentiation in tissue engineering approaches. In this case, DNA codifying growth and differentiation factors can be used and be released from the film along time, being internalized by cells^{372,382,500}. The *in situ* delivery of DNA by gene-activated matrixes is thus a

possible application for these films. Also here, drugs can be encapsulated within the polymer/DNA layers and suffer a simultaneous controlled release.

The following graphical summary captures everything that has previously been discussed, in terms of the type and methods of the most common DNA films develop to this day.



4. Conclusions and future perspectives

DNA has proven its important role in life continuity. However, its physical and chemical properties, easy manipulation, as well as easy obtainment, has now also made it an excellent candidate as a starting material in the materials field. Researchers all over the world have taken advantage of DNA's versatility as a structural and/or functional entity and have used it as a key material in hydrogels and films for several different applications. In the biomedical field, in particular, the application of DNA is made possible as it is a natural material that is water soluble, has high affinity for specific molecules and has biocompatibility and biodegradability properties.

DNA hydrogels have experienced the effects of time since 1987, in terms of the evolution and complexity of the different strategies involved in their preparation. Significant progress has been made by integrating active DNA entities such as aptamers, i-motifs, G-quadruplexes and DNAzymes, transforming simple hydrogels into smart or intelligent soft materials capable of undergoing volume or phase transitions that are essential in a delivery or detection system. In addition, the combination of these different DNA entities may result in an orthogonal-tunable behaviour (response to several different stimuli). Most of the research papers reported in the literature address bulk DNA hydrogels, with very few examples discussing their micro and nano counterparts. One hopes that the future brings continuous efforts, research and improvement of DNA hydrogels at the nanoscale, for possible systemic and targeted delivery of hydrophobic drugs or sensitive molecules, as well as for therapeutic and/or theranostic applications.

As DNA has shown itself to have a key function in stimuli-responsive hydrogels, it has also traced its path in ultrathin and thin films. DNA immobilization over a substrate has evolved from thicker cast films to chemisorbed monolayers (SAMs) having a very well-organized structure. Other methods using specific chemical reactions (e.g. through the use of heterobifunctional crosslinkers) or just physical interactions (e.g. LbL, LB) allowed the production of thin and ultrathin films. As these thin film deposition techniques are simple, versatile and relatively cheap, with endless possible combinations of surfaces (gold, glass, planar, colloidal, etc.) and molecules (polymers, crosslinkers, inorganic, organic, biomolecules, particles), its popularity is understandable. One can clearly see that films have evolved from thick and bulk cast films to thin/ultrathin layers. Once again, miniaturization has been the driving force for researchers to evolve and fulfill modern day society's needs and demands regarding technology, economy, environment and health, to improve our overall quality of life.

Chapter II

DNA/dendrimer-based films with potential biomedical applications

Abstract

Novel hybrid DNA films were prepared by heating double stranded DNA (dsDNA) above its melting temperature and, while the system was still hot (99°C), adding poly(amidoamine) (PAMAM) dendrimers. These DNA/dendrimer-based films were formed through electrostatic interactions established between the negative DNA molecules and the positively charged dendrimers, as well as through partial hybridization of the single stranded DNA (ssDNA) during the cooling process. The produced films were water-insoluble, almost transparent and highly stable over long time periods.

As the films were only formed under strict experimental conditions, the work started by the optimization of the process for their preparation. The formation of these new DNA/PAMAM dendrimer films was dependent on the DNA heating temperature, cooling speed, initial pH, buffer composition, DNA size/shape, dendrimer generation and N/P (amine/phosphate) ratio. Beyond PAMAM dendrimers (generations 3 to 5), films could also be produced with branched poly(ethylenimine) with 25 kDa.

Films were characterized to confirm the presence of DNA and PAMAM dendrimers in their structure using fluorescence labels. Films' observation by scanning electron microscopy (SEM) showed a homogeneous surface that seemed to contain interconnected spheres laying down over a more compacted layer of material. Also, surface zeta potential values were positive and circular dichroism analysis revealed evidences of supramolecular chirality. Although the produced films did not promote cell adhesion, it was possible to culture cells in their presence. The functionalisation of the films with cyclic arginine-glycine-aspartic acid (RGD) peptides, known for their specific interaction with integrins existent at the cell membrane, improved cell adhesion. At the end, the films were tested as platforms for the delivery of three anticancer drugs, namely 5-fluorouracil (5-FU), cisplatin (cisPt) and doxorubicin (DOX), which have distinct forms of interacting with DNA. The results revealed that these films were only interesting platforms for the delivery of doxorubicin, which is a DNA intercalating drug.

Keywords: DNA; PAMAM dendrimer; DNA film; electrostatic interactions; doxorubicin; cisplatin; drug delivery; RGD.

1. Introduction

As reviewed in Chapter 1, DNA has been a very important molecule in Materials Science, especially for the preparation of hydrogels and thin films. It has been widely explored because of its unique physical, chemical, and structural properties, such as: a tridimensional structure consisting of a double helix (stabilized by nucleobase pairing)⁵⁰¹; base pair complementarity²²; a hydrophilic and negatively charged phosphate backbone and hydrophobic nucleobase pairs¹⁴; double helix charge conductivity along nucleobase stacking⁵⁰²; and ability to exist as single strand DNA (ssDNA) after denaturation²¹. Other DNA tridimensional structures also exist, like triplexes, G-quadruplexes, i-motifs, DNAzymes, etc.⁶⁸. Indeed, DNA has shown great versatility and was used with success by many researchers working in inter- and multidisciplinary scientific subjects.

Dendrimers are hyperbranched molecules that are organized in layers (called generations) (Figure 58, A). They have well-defined size (are monodisperse) and molecular weight, and many terminal groups (are multivalent) that can be modified or functionalised (Figure 58, B). PAMAM dendrimers were discovered by Tomalia *et al.*⁵⁰³ in 1985. Their synthesis is a two-step process, through Michael addition reactions, where an amine initiator core reacts with a methylacrylate, followed by amidation of the esters with ethylenediamine⁵⁰⁴. The repetition of these steps allows for the exponential growth of the dendrimer which increases in molecular weight and double their terminal groups in each generation built. Their synthesis can follow two possible routes (Figure 58, C), namely, a divergent (when the dendrimer grows from the core) or a convergent (dendrons are first prepared and then attached to a core) route, each with their own advantages and disadvantages^{503,504}.

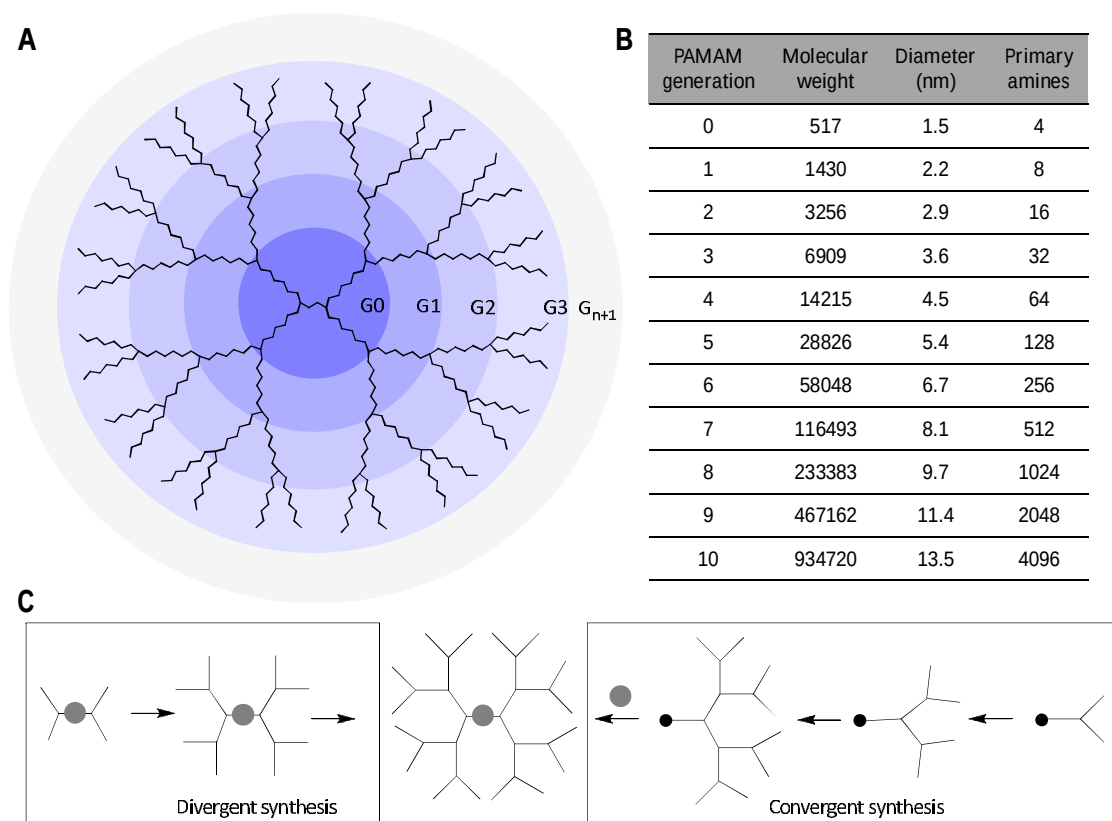


Figure 58 - PAMAM dendrimer physical and structural properties. (A) Two-dimensional representation of polyamidoamine (PAMAM) generation growth, with each branching point defining each layer. (B) PAMAM generations and corresponding molecular weight, diameter and number of surface primary amines, showing a double exponential growth (Adapted from Dendritech®^{505,506}). (C) PAMAM dendrimer divergent and convergent synthesis approaches.

PAMAM dendrimers (Figure 59, A) have an ethylenediamine (EDA) core, tertiary amines at the branching points and terminal primary amines at the surface, being molecules with a hydrophilic character. Furthermore, the molecular architecture of the dendrimers endows them with internal cavities and channels⁵⁰⁷ formed by the hydrophobic chain segments, conferring them an internal hydrophobic nature⁵⁰⁸. In Figure 59 (B-D), PAMAM generation (G3, G4 and G5) molecular structure are represented respectively.

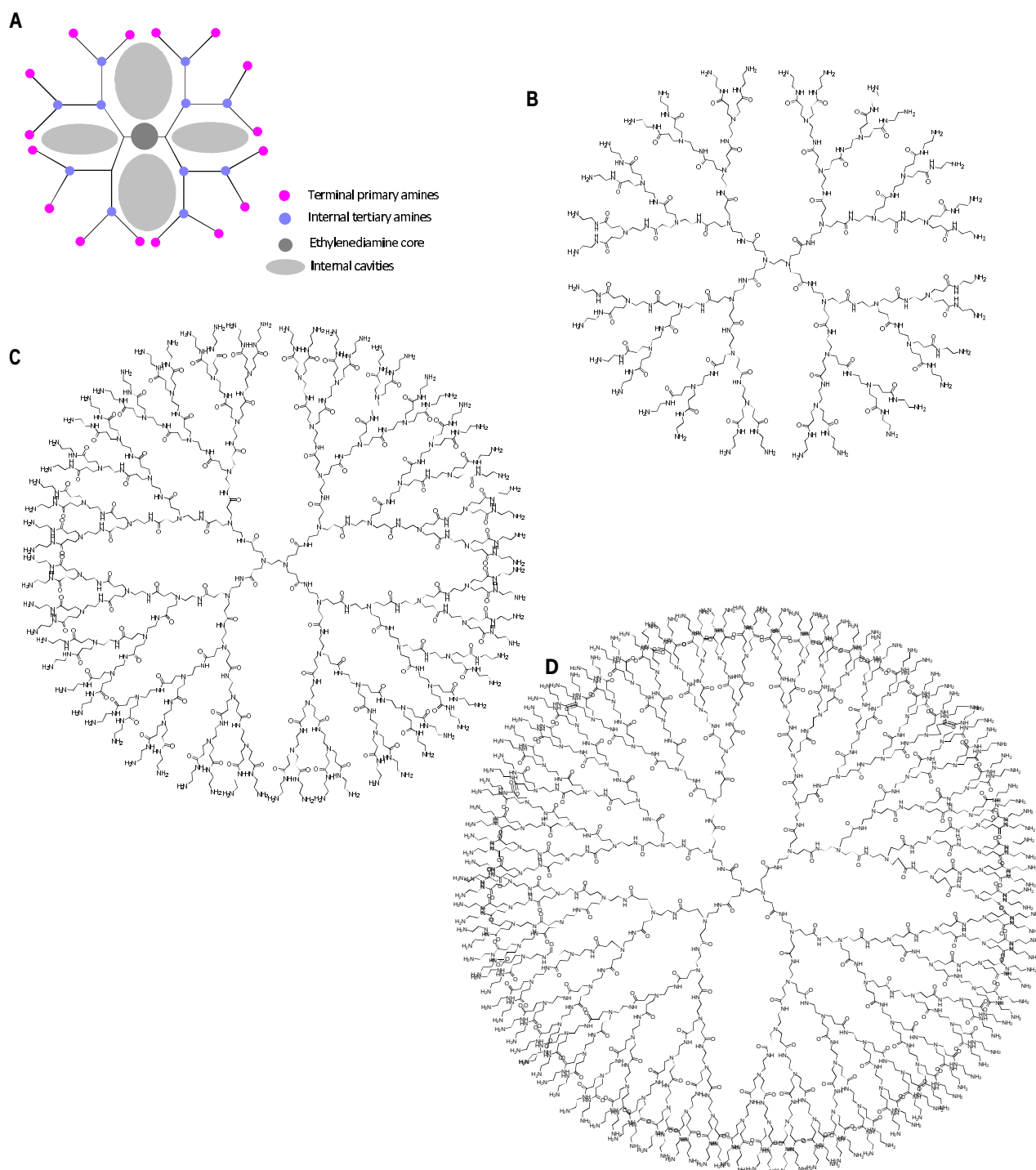


Figure 59 - PAMAM dendrimer structural representation and different generations. (A) Two-dimensional schematic representation of a G2 PAMAM dendrimer, with terminal primary amines at the surface, internal tertiary amines and inner cavities. Two-dimensional representation of the molecular structure of several polyamidoamine (PAMAM) dendrimer generation, namely (B) G3, (C) G4 and (D) G5.

Due to the existence of the inner tertiary amines and the surface functional groups, PAMAM dendrimers can present different protonation degrees (Figure 60), which are pH-dependent, resulting also in conformational changes^{509,510}. The internal tertiary amines have a pKa of around 4,

while the primary amines present a pK_a of around 7⁵⁰³. Maiti *et al.*⁵¹⁰ demonstrated that in high pH solutions (> 12) PAMAM dendrimers are not protonated, in neutral pH solutions ($= 7$) all primary amines are protonated and at low pH values (< 4) tertiary amines are fully protonated, making them strongly cationic polymers.

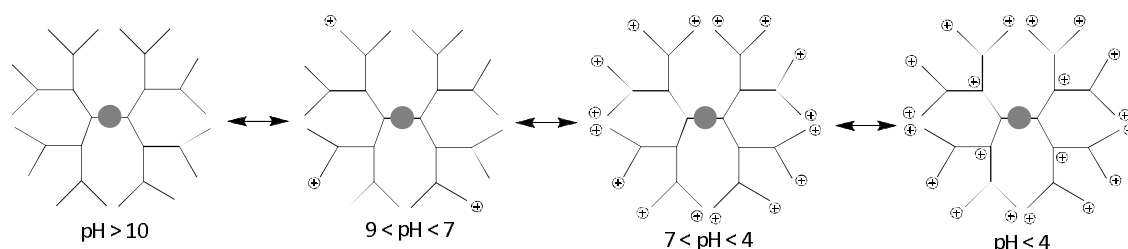


Figure 60 - PAMAM dendrimer pH-dependent protonation degree. At high pH, PAMAM dendrimer is neutral, and as the pH decreases, the primary and tertiary amines get protonated in this order. The process is reversible.

Due to all these physical, chemical and architectural properties, PAMAM dendrimers have been used for many applications⁵¹¹, especially for drug^{512–514} and gene^{500,515–520} delivery (Figure 61). This is only possible by taking advantage of PAMAM's protonation to establish ionic electrostatic interactions with the negatively charged nucleic acids^{521–523} or PAMAM's cavities to load/encapsulate drugs^{524–526}, despite some of its toxicity effects attributed to their positive charge at the physiological pH⁵²⁷. Many papers about PAMAM dendrimers, addressing synthesis and functionalisation^{528–531} to applications^{532–534}, have been published since 1985.

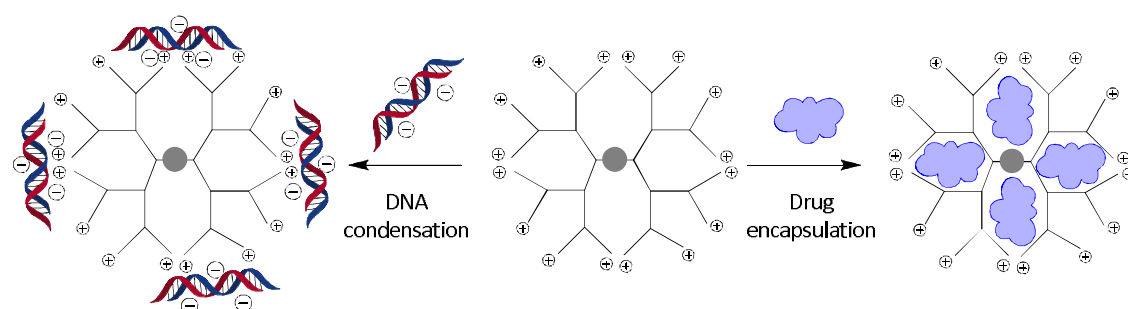


Figure 61 - PAMAM dendrimer in drug and gene delivery applications. DNA condensation is driven by electrostatic interactions between the opposite charged molecules, while the PAMAM dendrimer can be a host for small molecules such as anticancer drugs.

In fact, our research group has been working for some years in the dendrimer field, namely in the synthesis of new metallodendrimers^{535–540} and, especially, in the functionalisation of dendrimers^{541–546} for gene and drug delivery applications. So, based on the expertise of the group in dendrimer chemistry and knowing that these molecules have an intrinsic tendency to interact with DNA, we raised the hypothesis of preparing macroscopic three-dimensional (3D) hydrogels from

these two components. For that purpose, instead of using plasmid DNA (pDNA), as is usual in gene delivery studies, a linear and long DNA was selected for hydrogel preparation. The original idea to create DNA/dendrimer hydrogels was to use the linear DNA as building block for the tridimensional structure and PAMAM dendrimers has electrostatic crosslinker. The experimental approach would be to promote DNA denaturation by increasing its temperature above its melting temperature (T_m), in order to obtain single stranded (ssDNA). At this point, the introduction of the dendrimers in the system would lead to electrostatic interactions with the ssDNA. Afterwards, the cooling process would also promote random hybridization between free ssDNA strands. This way, the tridimensional structure would be supported by the DNA/dendrimer electrostatic interactions and the nucleobase hydrogen bonds. Unexpectedly, when the process was followed by slow cooling, a soft film appeared in the bottom of the tube. As such, in place of 3D hydrogels, two-dimensional (2D) hybrid films were obtained which represent a new class of DNA/dendrimer materials. Materials like these ones have never been reported in the literature.

Therefore, this work will describe in detail the experimental conditions necessary for the preparation of hybrid DNA/dendrimer films, as well as their physicochemical characteristics. The fact that these films are only formed under very specific conditions provided clues as to the nature of the interactions between the chemical species involved. Hybrid DNA/poly(ethylenimine) (DNA/PEI) films were also successfully obtained using the same methodology. Since these materials are envisaged to be applied in the biomedical field, their stability under conditions that mimic the physiological environment was studied. As well, the possibility to support cell growth under their surfaces was evaluated and improved by conjugation with arginine-glycine-aspartic acid (RGD) peptides. Finally, these systems were assayed as platforms for the delivery of anticancer drugs, namely doxorubicin (DOX), cisplatin (cisPt) and 5-fluorouracil (5-FU).

As seen before, PAMAM dendrimers have been used in films, and specially in DNA biosensor, with covalently bond dendrimer and DNA onto a surface^{343–346}. Here we report the production a new material, a hybrid DNA film, with DNA and PAMAM dendrimers (also with PEI) based in electrostatic interactions using a different methodology, not yet reported in the literature. Elucidation of the specific experimental conditions in the successful preparation of these novel DNA films was pursued. Characterization and stability studies were performed, as well as cell biocompatibility, DNA binding drug accumulation and cumulative release.

2. Experimental section

2.1 Materials, cells, reagents and equipments

Deoxyribonucleic acid sodium salt from salmon testes (SS-DNA) in dry state was acquired to Sigma-Aldrich (Merck). According to the supplier, it is a long double-helical molecule with %G-C content of 41.2%, T_m (melting temperature) of 87.5°C (in 0.15 M sodium chloride plus 0.015 M sodium citrate) and with an approximate molecular mass of 7.8×10^6 Da ($\approx$ 12 kbp; as determined in the present work by agarose gel electrophoresis). Branched poly(ethyleneimine) (brPEI, 0.8 and 25 kDa), linear PEI hydrochloride (lnPEI-HCl, 10 kDa), poly(allylamine hydrochloride) (PAH-HCl, 56 kDa), chitosan oligosaccharide lactate (COL, 5 kDa), protamine from salmon (4.2 kDa, grade IV, histone free), rhodamine B isothiocyanate (RITC), Dulbecco's Phosphate Buffered Saline (PBS, cat # D5773, without calcium), resazurin, imidazole, 6-maleimidohexanoic acid N-hydroxysuccinimide ester (EMCS), imidazole, N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC-HCl), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), deuterium oxide (D_2O , 99.8 atom % D), agarose powder, tris(hydroxymethyl)aminomethane (Tris), *ortho*-phenylenediamine (OPDA), GenElute Endotoxin-free Plasmid Maxiprep Kit and bicinchoninic acid (BCA) protein quantification assay kit were purchased from Sigma-Aldrich (Merck) too. Plasmid DNA (pDNA) encoding enhanced green fluorescent protein (EGFP) (pDNA-EGFP, 6.4 kbp) was amplified and extracted from transformed DH5 α *E. coli* using a DNA extraction kit. Quantification and purity was determined by UV-vis absorbance at 260/280 nm. Generations 1, 2, 3, 4 and 5 poly(amidoamine) (G1, G2, G3, G4, and G5 PAMAM) dendrimers with ethylenediamine (EDA) core and terminal amine groups ($-NH_2$), dissolved in methanol, were purchased from Dendritech Inc.. Pre-treated regenerated cellulose dialysis membranes with molecular weight cut-off (MWCO) of 0.5-1, 1, 3.5, 6-8 and 12-14 kDa were from Spectrum® Labs. Dulbecco's Modified Eagle's Medium - high glucose (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI 1640), foetal bovine serum (FBS), antibiotic-antimycotic (AA), L-glutamine (L-Glut) and trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA) were from Gibco® (ThermoFisher Scientific). Quant-iT PicoGreen® dsDNA Assay Kit was purchased from ThermoFisher Scientific. Dimethylsulfoxide (DMSO), glacial acetic acid (HAc), dimethylformamide (DMF), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA-Na), sodium chloride (NaCl) and hydrochloric acid (HCl) were purchased from Fisher Scientific (ThermoFisher Scientific). Potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4) and sodium dihydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) were obtained from Panreac. Sodium hydroxide (NaOH) was purchased from Merck. cis-diaminoplatinum (II) dichloride (cisplatin, cisPt) was purchased from Acros

Organics, 5-fluorouracil (5-FU) from TCI Chemicals and sodium hydrogen carbonate (NaHCO_3) from LabChem. The cyclic RGD peptide (c(RGDFK)-Cys, cRGD) (MW = 708.6 g/mol) was purchased from GenicBio, amine-poly(ethylene glycol)-carboxylic (NH_2 -PEG-COOH, 2 kDa) from YareBio and doxorubicin hydrochloride (DOX-HCl) from Zibo Ocean International Trade Co. Ltd.. Ethidium Bromide (EtBr) at 10 mg/mL in water was purchased from MPBio. DNase/RNase-free water (HyPure Molecular Biology Grade Water) was from GE Healthcare Life Sciences. Blue loading buffer and GeneRuler™ 1kb DNA ladder were purchased from Fermentas (ThermoFisher Scientific).

All aqueous solutions were prepared with 0.22 μm filtered ultrapure water (UP H_2O) from Milli-Q® Direct 8 water purification system from Merck Millipore (Merck) and subsequently filtered with a 0.20 μm cellulose acetate filter (VWR). pH adjustments and measurements were done using a SympHony SB21 pH Meter (VWR) adapted with a LoT406-M6-DXK puncture pH electrode (Mettler Toledo) in order to fit into micro- and centrifuge tubes. Films prepared in glass flasks were heated in the incubators model UM200 (Mettler GmbH) and model T6030 (Heraeus), while the ones prepared in 2 mL microcentrifuge tubes (SC - SuperClear™, VWR) were heated using a 1.5 mL ThermoStat™ Plus thermoblock (Eppendorf). The stability and release studies were performed in a WIS-30 shaking incubator (Wisd Laboratory Instruments). NIH 3T3 (mouse embryo fibroblast cell line), A2780 (human ovarian carcinoma cell line), and A2780cisR (human ovarian carcinoma cell line resistant to cisplatin) cells were acquired from DSMZ GmbH (Germany) and ECACC (UK). Cell culture manipulation and growth were done in a vertical laminar airflow safety cabinet (class II, NuAire) and an Autoflow IR Direct Heat CO_2 incubator (NuAire), respectively. Millicell hanging cell culture inserts - transwells (cat # MCRP24 h4, PET, 1.0 μm pore size) for 24-well plates were purchased from Merck Millipore. UV-visible measurements were made in a Lambda 25 UV-vis spectrophotometer (PerkinElmer®). Different ss-DNA sizes were achieved by sonication in an ultrasonic bath 2510 (Branson), 30 s ON (bath) / 30 s OFF (ice), stopping sonication every 10 min, in a total of 70 min (7 tubes). Film and cell culture bright field and fluorescence images were taken with an Eclipse TE 2000E fluorescence inverted microscope (Nikon Instruments). Cytocompatibility (resazurin reduction assay) was evaluated using a Victor³ 1420 microplate reader (PerkinElmer). NMR spectra were obtained using an Avance II+ 400 MHz NMR spectrometer (Bruker). Lyophilization was carried out in a FreeZone 4.5 Liter Console Freeze Dry System (Labconco®). Scanning electron microscopy (SEM) images were obtained using a FEG-SEM (JEOL 7001F) and a benchtop SEM (Phenom - Pro X). Circular dichroism (CD) measurements were performed with a J-815 Circular Dichroism (CD) Spectropolarimeter coupled with a Peltier Type Cell (PTC)-423S (Jasco Inc.). Surface zeta-potential measurements were obtained in a ElectroKinetics Analyzer (EKA) (Anton Paar). Stained agarose gels were run in a EC Minicell Primo EC-320 horizontal gel system (Thermo Scientific) and documented in a GelDoc™ EQ Imaging System Model 170-8060 (BioRad).

2.2. Optimizations of the experimental conditions for the preparation of DNA/dendrimer films

The general protocol for preparing DNA/dendrimer films was composed by the next simple steps: SS-DNA was mixed with a salt solution at an appropriate concentration (1 mg/mL) and heated; at this point, the dendrimers were added, and the system was left to cool down, allowing for the film formation. Several experimental conditions essential for DNA/dendrimer film formation were studied by varying the solution composition (H_2O , 0.15 M NaCl, 1x TE buffer, 1x PBS), the concentration and size/shape of DNA, the initial pH (5, 6, 7 and 8) of the SS-DNA solution, the dendrimer's generation, the maximum heating temperature (no heating, SS-DNA's $T_m = 87^\circ\text{C}$ and 99°C) and the cooling rate (on ice (0°C), at room temperature (RT) or using a controlled temperature ramp (CTR)). All these conditions were optimized using G3, G4 and G5 PAMAM dendrimers. Several N/P ratios (0 to 10) were tested (the N/P ratio is the molar ratio between the number of amine groups in the dendrimers and the number of phosphate groups in the DNA). The experimental details used in these studies are presented in the next paragraphs.

2.2.1. Dendrimer, SS-DNA and salt solutions

First, in order to use PAMAM dendrimers, methanol had to be removed from the acquired dendrimer solutions. Dialysis against water was performed for 3 days using regenerated cellulose dialysis membranes with the appropriate MWCO for each generation, followed by lyophilization. Stock solutions of each PAMAM dendrimer were prepared in filtered UP H_2O at a concentration of 50 mg/mL.

The size of the acquired SS-DNA was approximately 12 kbp, as revealed by the several agarose gel electrophoresis done along this work. A SS-DNA stock solution was prepared by addition of filtered UP H_2O to achieve a concentration of 10 mg/mL. This solution was vortexed and left for 24 h at 4°C to allow for the complete dissolution. At this concentration, the resulting solution was highly viscous.

A PBS stock solution (10x PBS) was prepared with UP H_2O with the following composition: 8.00 g NaCl, 0.20 g KCl, 0.24 g KH_2PO_4 and 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in a final volume of 100 mL, pH = 6.8, followed by filtration. A Tris-EDTA buffer stock solution (10x TE) was prepared at 100mM Tris-HCl and 10 mM EDTA, pH = 7.5.

2.2.2. Established protocol for DNA/dendrimer film preparation

After optimizing the experimental conditions for film preparation, a protocol was established for the subsequent studies. Assays were done using a final SS-DNA concentration of 1 mg/mL. For starting, 50 μ L of a 10 mg/mL SS-DNA stock solution (500 μ g of sodium salt SS-DNA) were added to 2.0 mL SC tubes. Afterwards, for a final volume of 500 μ L, 50 μ L of 10x PBS solution (final concentration of 1x) were added, as well as the adequate volume of UP H₂O (taking in account the volume of dendrimer solution later added which was dependent on the desired N/P ratio). The pH of the previously prepared DNA solution (SS-DNA + PBS + UP H₂O) was then adjusted with the necessary amount of 5 M NaOH or 6 M HCl, from the initial value (usually 6.95 ± 0.10) to 6.00 ± 0.05 , using the pH meter (previously rinsed with distilled H₂O, ethanol and dried). In a separated SC tube, the volume of dendrimer solution needed to obtain the desired N/P ratio was aliquoted. The SS-DNA and the dendrimer solutions (tubes closed) were separately heated at 99°C for 10 min using the thermoblock. The two solutions were then mix and homogenized by "up and down" fluid motion using the micropipette. The tubes were kept at 99°C for 5 min and then allowed to cool down using the thermoblock from 99°C to 87°C, 68°C, 50°C and 24°C, stopping 5 min at each of these temperature points. The presence of the film was confirmed visually and by touching it with the micropipette tip. When formed, the films could be detached from the bottom of the tube in one piece. This optimized process for film preparation is schematized in Figure 63. All films prepared using these conditions had approximately 472 μ g of SS-DNA per cm² of film surface.

2.2.3. Solution' final pH after SS-DNA/dendrimer film preparation

The optimal pH of the initial SS-DNA solution (SS-DNA + PBS + UP H₂O) was determined by adjusting its pH to different values (5.00, 6.00, 7.00 and 8.00 (± 0.05)) and observing film formation. The change in the final solution pH resulting from dendrimer solution addition was evaluated too. Briefly, the general protocol for film formation was followed using G5 PAMAM dendrimers at different N/P ratios (0 to 10) in separated 2.0 mL SC tubes. Several sets of SS-DNA solution were prepared and the pH of the solutions was adjusted to the intended value with HCl (6M) or NaOH (5M) solutions. After heating the SS-DNA and dendrimer solutions for 10 min, at 99°C, the necessary amount of dendrimer was added to each tube, and the system was cool down in the thermoblock. After this process, the pH of the solutions was measured. Further experiments were made with G3 and G4 PAMAM dendrimers but always starting from an initial pH of 6.00. The electrode was always washed with UP H₂O and ethanol, and dried before and between measurements. Several controls were performed, namely, without dendrimer, without SS-DNA and without both (always substituting

the volume of missing solution with water). Results are present as mean $\pm$ standard deviation (s.d.), with $n = 3$.

2.2.4. Agarose gel electrophoresis assay

Since SS-DNA/dendrimer films were not formed when a fast cooling process was applied or in the presence of magnesium ions, the effects of these two parameters on SS-DNA molecule conformation, and on the hybridization process after SS-DNA heating (above its T_m temperature) and cooling, were investigated by agarose gel electrophoresis assays. For the agarose gel electrophoresis experiments, solutions containing 2 μg of SS-DNA in 1x PBS (with and without Mg^{2+}) were prepared in a final volume of 16.7 μL . Part of these solutions were heated and subjected to different cooling processes (on ice (0°C), at room temperature (RT) or using a controlled temperature ramp (CTR)). Samples that did not pass through the heating/cooling steps (control samples) and samples containing SS-DNA mixed with G5 PAMAM dendrimers at a N/P ratio of 4 were also prepared. These last samples were also subjected to different heating/cooling processes. Afterwards, 3.3 μL of Blue Lading Buffer were added to each sample to obtain a final volume of 20 μL . From each of the prepared solutions, 10 μL were loaded into 1% (w/v) agarose gel in 1x TAE (48.4 g Tris-base, 1.142 mL glacial acetic acid and 2 mL of 0.5M EDTA (pH = 8)) with 0.06 μL of 10 mg/mL EtBr per mL of gel. The electrophoresis was carried out at 75 V, for 30 - 45 min.

2.2.5. PicoGreen® assays

A study of the effect of magnesium ion's presence (0.5 mM) on the electrostatic interactions established between SS-DNA and dendrimers was also done by evaluating the ability of dendrimers to compact the SS-DNA using the PicoGreen® assay. DNA compaction by G5 PAMAM dendrimers was analysed using the Picogreen® assay. To do so, SS-DNA and G5 PAMAM dendrimer stock solutions were prepared at concentrations of 0.01 mg/mL and 0.05 mg/mL, in filtered UP H_2O , respectively. The Picogreen® assay was always done at a constant SS-DNA concentration of 0.1 $\mu\text{g}/\text{mL}$. Having this in mind, 10 μL of SS-DNA were added to each test tube, followed by the addition of adequate volumes of 10x PBS and filtered UP H_2O . Afterwards, adequate volumes of G5 PAMAM dendrimer solutions were added to get N/P ratios from 0 to 10. PBS solutions with and without magnesium ions were used. The final volume in the test tubes was always 100 μL . After 30 min incubation at RT, 100 μL of the test solutions were transferred to opaque 96-well plates and 100 μL of Picogreen solution (dilution 200x, in 1x TE buffer, prepared according the supplier's instructions⁵⁴⁷) were added to each tube. After incubation for more 5 minutes, fluorescence was measured in a microplate reader, with

λ_{ex} = 485 nm and λ_{em} = 535 nm. Additional assays were also performed where the SS-DNA solutions and the dendrimer solutions were separately heated at 99°C for 10 min, mixed and then cooled until RT using a controlled temperature ramp, following the same procedure used for film preparation. The percentage of free DNA in solution (not condensed by the dendrimer) was calculated using the following equation:

$$\% \text{ free DNA} = \frac{F_{NP}}{F_{NP=0}} \times 100 \% \quad (1)$$

where F_{NP} corresponds to Picogreen's fluorescence at each N/P ratio above 0 and $F_{NP=0}$ corresponds to Picogreen's fluorescence at N/P ratio 0 (control). Results are presented as mean $\pm$ s.d., with $n = 3$.

2.3. Preparation of DNA films using other polymers

DNA/dendrimer films were first successfully prepared with G3, G4 and G5 PAMAM dendrimers and these were the polymers used for optimizing the process of film formation (see the previous sections). However, the possibility of creating films using lower generation PAMAM dendrimers and other polymers was also tested. For these studies, the following molecules were used: G1 and G2 PAMAM dendrimers, linear poly(ethylenimine) (lnPEI-HCl - 10 kDa), branched poly(ethylenimine) (brPEI - 0.8 and 25 kDa), linear poly(allylamine) hydrochloride (PAH-HCl - 56 kDa), linear chitosan oligosaccharide lactate (COL - 5 kDa) and protamine from salmon (4.2 kDa). All stock solutions prepared from these molecules were made at a concentration of 50 mg/mL and allowed to solubilize for 24 h at 4°C. Film preparation was done in 2 mL SC tubes, following the previously established optimized conditions (pH = 6, in 1x PBS) and using appropriate volumes from each polymer stock solution to achieve a N/P ratio of 4. After the cooling process, the presence of the film was confirmed visually and by touching it with a micropipette tip and seeing its detachment from the bottom of the tube.

2.4. Characterization of the DNA/dendrimer films

2.4.1. Presence of SS-DNA inside the films

To confirm the presence of SS-DNA inside the films, a film based on SS-DNA and G4 PAMAM dendrimers, at a N/P ratio of 5, was prepared. The same methodology previously described was followed but, this time, the film was prepared inside a typical clear chromatography glass vial and an incubator was used to heat the solutions and cool down the system until 24°C (for approximately 6 h). After obtaining the film, the surrounding solution (supernatant) was removed and the film was washed with 1 mL of UP H₂O. Then, it was stained using 1 mL of a 20 µM solution of EtBr in H₂O for 24 h, protected from light. Afterwards, the staining solution was removed and the film rinsed with water. The color change was visually detected and fluorescence emission was confirmed by placing the vial with the EtBr stained film under the blue light (330 - 380 nm) of a fluorescence inverted microscope.

2.4.2. Presence of PAMAM dendrimers inside the films

To confirm the presence of PAMAM dendrimers inside the films, G4 PAMAM dendrimers labeled with the fluorescence dye RITC (G4-RITC) were used for film preparation. First, lyophilized G4 PAMAM dendrimer (163.90 mg; 0.115 µmol) was dissolved in 4.5 mL DMSO. A 1.5x molar excess of RITC powder (9.94 mg; 0.173 µmol) was also dissolved in 0.5 mL DMSO. The latter was added drop-by-drop to the dendrimer solution and left to react for 24 h under vigorous stirring, at room temperature (RT). Dialysis was performed for 3 days, against water, using a 6-8 kDa MWCO dialysis membrane, and the solution containing the functionalised dendrimer was then freeze-dried for 3 days. The number of RITC molecules per dendrimer was determined using a UV-vis spectrophotometer and a RITC standard calibration curve in water. Results showed that the ratio RITC:dendrimer was 1 (1.01 RITC : 1.00 dendrimer). A film was then prepared (in a chromatography glass vial) using the labeled dendrimer and SS-DNA, at a N/P ratio of 5, and using the optimized conditions previously described. Afterwards, the solution surrounding the film was removed and the film was washed with 1 mL of UP H₂O. At the end, the film was kept in 1 mL of fresh UP H₂O. The color change was visually detected and the fluorescence emission was confirmed by placing the vial with the labeled film under the red light (510 - 560 nm) of the fluorescence inverted microscope.

2.4.3. Stability studies under extreme pH values

The stability of the films was evaluated in extreme pH values (alkaline and acid extreme values). To do this, several films were prepared using SS-DNA and G5 PAMAM dendrimers (N/P ratio 0 to 10). Briefly, two sets of films were prepared in 2.0 mL SC tubes (one set for testing an extreme alkaline pH and the other for testing an extreme acid pH). The solutions' pH was measured using the methodology previously described. Then, 10 μ L of 1 M NaOH or 10 μ L of 1 M HCl was added to the correspondent set of films. Again, the pH value was measured and changes were recorded. Two other films were prepared (G5 dendrimer, N/P ratio 4), washed with 1 mL of UP H₂O, and placed in a glass coverslip. After, 10 μ L of 6 M HCl was added to one of the films and 10 μ L of 5 M NaOH was added to the other. These films were observed in an inverted microscope starting from the moment of addition of the acid or the base.

2.4.4. Scanning electron microscopy (SEM)

SEM analysis was performed with lyophilized samples. Several films based on G3 and brPEI, at a N/P ratio of 4, were prepared on glass coverslips with $\varnothing = 1.3$ cm. After, the films were washed twice with 2 mL of filtered UP H₂O and lyophilized. After sputtering the samples with gold, images were recorded in a FEG-SEM equipment, at accelerating voltages of 15 - 20 kV, under high vacuum. Magnification was from 100 to 10,000x. Films were also analysed in a benchtop SEM using a charge reduction sample holder and an accelerating voltage of 10 kV.

DNA films based on G3 PAMAM dendrimers, at a N/P ratio of 4, and irradiated using UV light were also prepared to be observed by SEM. After washing with 2 mL of filtered UP H₂O, films were exposed to UV radiation (in the vertical laminar flow chamber) for 6 h, 24 h and 48 h. These films were also lyophilized prior observation and gold sputtered.

2.4.5. DNA/dendrimer film's stability studies under physiological conditions

The degradability of DNA/dendrimer films was evaluated *in vitro* using six solutions (degradation solutions) mimicking the physiological environment, namely: DNase/RNase-free H₂O, 1x PBS (pH = 7.4); DMEM (pH = 7.4); 1% FBS (in 1x PBS), 10% FBS (10% in 1x PBS) and complete DMEM (DMEM with 1% AA and 10% FBS). For these experiments, films prepared with G3, G4 and G5 PAMAM dendrimers, as well as with brPEI25, were prepared at a N/P ratio of 4. Assays were conducted at 37°C, and the released DNA was quantified at different time points.

The exact amount of SS-DNA present in each film before the degradation studies was always determined. Briefly, after preparing the film and working in the vertical laminar flow chamber to avoid contaminations, the supernatant resulting from film formation was homogenized and 300 μL were withdrawn to a 1.5 mL microcentrifuge tube containing 700 μL of UP H_2O (the blank consisted in 300 μL of 1x PBS and 700 μL of UP H_2O). Absorbance at 260 nm was measured to quantify the initial amount of SS-DNA in the film (by subtracting the achieved value from the initial amount of SS-DNA used to prepare the film). Then, for the degradation assays, the remaining supernatant was discarded, the film was washed with 1 mL of filtered UP H_2O and 1 mL of the degradation solution was added. The film in contact with the degradation solution was placed at 37°C, inside the incubator, without shaking. Blanks without films were also prepared and kept in the same conditions. At different time points, solutions were homogenized with a micropipette, and 300 μL were removed (which were always replaced by 300 μL of fresh solution) to 1.5 mL microcentrifuge tubes with 700 μL of UP H_2O (the same procedure was performed with the blanks) and absorbance at 260 nm was measured.

The cumulative degradation ($\mu\text{g/mL}$) was calculated using the following equation:

$$\text{Cumulative degradation} = \frac{V_t}{V_0} \times D_{t-1} + D_t \quad (2)$$

where V_t corresponds to the withdrawn volume (0.3 mL), V_0 to the volume of solution (1 mL), D_t is the degradation obtained at the actual time point (μg) and D_{t-1} is the degradation obtained in the previous point (μg). Results are presented as mean $\pm$ s.d., with $n = 5$.

2.4.6. Surface zeta-potential (ZP) measurements

The zeta-potential of the DNA/dendrimer films was also analyzed. In this case, thicker and more handling resistant films had to be prepared. As such, DNA/dendrimer films (based on G3, G4 and G5 PAMAM dendrimers, at N/P = 3, 4 and 5) were prepared in $\varnothing = 2.2$ cm glass sample flasks using a triple amount of SS-DNA/ cm^2 (1416 $\mu\text{g}/\text{cm}^2$). After all the solutions were prepared as previously described, they were heated in the incubator at 99°C, for 1 h, and were left inside to cool down to 24°C. Surface zeta-potential measurements were performed using a ElectroKinetics Analyzer (EKA, Anton Paar). Briefly, each film was placed in a piece of parafilm and the sample holder was placed on top. Warmed paraffin wax (enough to be liquid) was injected into it, filling the gap between the film and the sample holder (this way the film could present a solid and flat surface). The sample holder with the film was then positioned in the equipment, and a poly(methylmethacrylate)

(PMMA) clamping cell was placed on the top of the film, with the micro channels facing the film. A solution of 1 mM KCl, pH = 5.5 ± 0.2 was used to measure two ZP values for each film (left and right flow direction), with a pressure of 200 mbar, at a 25°C. The measurements were also performed with a PMMA block, as a standard reference for correction. Three measurements were recorded for each sample. Results are presented as mean $\pm$ s.d., with n = 3.

2.4.7. Circular dichroism (CD) measurements

For circular dichroism measurements, DNA/dendrimer films (based on G3, G4 and G5 PAMAM dendrimers, at N/P = 3, 4 and 5) were prepared accordingly the optimized conditions, in 2.0 mL SC tubes. The films were then removed from the tube and transferred to a cuvette with detachable windows (QS, 0.1 ± 0.005 mm, 26 μ L, Hellma), as flat as possible, and transferred to the CD spectropolarimeter. Measurements were performed at 20°C, between 340 - 200 nm (with increments of 0.2 nm), standard sensitivity, DIT 0.2 s, 50 nm/min speed and 9 accumulation, without baseline correction.

2.5. Cytocompatibility studies

Cytocompatibility studies were performed using DNA films prepared with G3 PAMAM dendrimers, at a N/P ratio of 4, in glass coverslips. For that, the glass coverslips ($\varnothing$ = 1.3 cm) were previously cleaned in 1M HCl at 50 - 60°C for 4h, washed with filtered UP H₂O, sonicated for 30 min and finally autoclaved. Then films were prepared in $\varnothing$ = 2.74 cm glass sample flasks with a glass coverslip inside, using 472 μ g/cm² SS-DNA (considering the flask diameter). After obtaining the films and always working in the vertical laminar flow chamber to avoid any contaminations, the supernatants were removed and the films were washed twice with 2 mL of DNase/RNase-free water. The film-containing coverslips were then transferred to 24-well plates. One last wash with PBS (Sigma-Aldrich) was performed before cell seeding.

NIH 3T3 fibroblast cells were grown in DMEM supplemented with 10% FBS and 1% AA. Cells were incubated at 37°C, in a humidified atmosphere containing 5% CO₂. When cells reached 60%-80% confluency, they were washed with pre-warmed PBS solution and trypsinized with trypsin-EDTA for 5 min in the incubator. Then, cell culture medium was added and cell concentration was determined. Cells were seeded on the top of the films at a density of 5×10^5 cells/well, in a final volume of 1 mL of complete cell culture medium. Control experiments were done in wells without coverslips. Cell morphology and growth were observed in an inverted microscope (100x

magnification), 24 and 48 h after the starting of the experiments. Cell viability was also evaluated using a metabolic activity assay, namely the resazurin reduction assay. Briefly, cell culture medium was replaced by 1 mL of fresh complete medium containing 10% (v/v) of a 0.1 mg/mL resazurin solution. Cells were, then, incubated for 3 h, after which 100 μ L were removed and placed in an opaque 96-well plate. Resorufin fluorescence (λ_{ex} = 530 nm, λ_{em} = 590 nm) was measured using a microplate reader. The percentage of viable cells was calculated by the following equation:

$$\% \text{ cell viability} = \frac{F_{\text{film}}}{F_{\text{control}}} \times 100 \% \quad (3)$$

where F_{film} corresponds to the fluorescence measured in the samples containing films and F_{control} corresponds to that measured in the control wells (without films and coverslips). Results are presented as mean $\pm$ s.d., with $n = 4$.

2.6. Film functionalisation with RGD peptides and evaluation of cell adhesion improvement

With the aim at improving cellular adhesion, the DNA/dendrimer films were functionalised with cyclic arginine-glycine-aspartic acid (cRGD) peptides. To do so, the methodology reported by He *et al.* was followed⁵⁴⁸. In more detail, cRGD peptide was first conjugated to NH_2 -PEG-COOH (spacer) via a thioether bond, using the heterobifunctional crosslinker EMCS at a molar ratio of 1:1:1. Briefly, NH_2 -PEG-COOH (141.48 mg, 0.071 mmol) was dissolved in 1 mL of 0.1 M imidazole buffer (pH = 6) and placed under vigorous stirring (1250 rpm) at RT. Next, EMCS (21.81 mg, 0.071 mmol) in 1 mL of DMSO was added dropwise and the mixture was left to react for 2 h (RT, 1250 rpm). Afterwards, the cRGD peptide (50 mg, 0.071 mmol) was dissolved in 2 mL of imidazole buffer and added to the EMCS-PEG-COOH solution. The reaction continued for further 24 h in the same conditions, after which dialysis was performed with a MWCO 1kDa dialysis membrane for 4 h (5 x 1 L of distilled H_2O , under agitation), followed by lyophilization for 3 days in order to obtain the RGD-PEG-COOH. The product was then characterized by ^1H NMR after dissolution in D_2O . The NMR results showed that the achieved modification was 1 RGD-PEG-COOH : 12 NH_2 -PEG-COOH which was in agreement with the literature⁵⁴⁸. Afterwards, sixteen DNA films were separately prepared in $\varnothing = 2.74$ cm flask with a $\varnothing = 1.3$ cm glass coverslip, based on G3 PAMAM dendrimers, at a N/P ratio of 4. After obtaining the films and always working in the vertical laminar flow chamber, the film-covered glass coverslips containing the films were placed in two 24-well plates (one to be sacrificed after 24 h of cell culture and the other to be sacrificed after 48 h), washed with 0.5 mL DNase/RNase-free water and kept at 4°C with

1 mL of the same water until the next day. Of the eight films placed in each 24-well plate, four of each plate were assigned as controls and the other four were functionalised with [cRGD-PEG + NH₂-PEG-COOH] : [G3 PAMAM dendrimers] at a molar ratio of 1.4 : 1. Having in considering the amount of G3 PAMAM dendrimer used to prepared each film, in the A = 5.91 cm² flask (Ø = 2.74 cm), 7.29 mg of G3 PAMAM dendrimer was added. Assuming that the dendrimers were uniformly distributed during the preparation process, in the A = 1.33 cm² flask (Ø = 1.3 cm coverslip), the amount of G3 PAMAM dendrimer is 1.64 mg (0.237 µmol). Briefly, [cRGD-PEG + NH₂-PEG-COOH] (7.788 mg, 2.69 µmol; for the eight films to be modified) was dissolved in 1 mL of filtered imidazole buffer (pH = 6) inside a 15 mL centrifuge tube. Next, EDC (0.86 mg, 4.48 µmol, 1.66x excess to cRGD-PEG + NH₂-PEG-COOH) in 0.25 mL imidazole buffer and sulfo-NHS (0.97 mg, 8.45 µmol, 3.14x excess to cRGD-PEG + NH₂-PEG-COOH) in 1 mL imidazole buffer were added to the cRGD-PEG + NH₂-PEG-COOH. The volume was completed to 8 mL by adding 5.75 mL of the buffer. Then, 1 mL of this mixture was added to each film and left to react for 2 h (for the controls, 1 mL of filtered UP H₂O was used in this step), after which the solution was removed and the films were washed twice with 1 mL of filtered UP H₂O. One last wash using 1 mL of PBS solution was performed and the films were left in the plate, with no solution, until NIH 3T3 cells were seeded on their top as described in the previous section. Again, optical microscopy was used to observe cell morphology and growth over time. Cell viability was also quantitatively evaluated in these functionalised DNA/dendrimer films using the resazurin reduction assay. Results are presented as mean ± s.d., with n = 3.

2.7. Studies on the used of DNA/dendrimer films as anticancer drug release platforms

2.7.1. Drug loading assays

The DNA films were exposed to solutions of different anticancer drugs (DOX, cisPt and 5-FU). Stock solutions the anticancer drugs were prepared in PBS solution and left to completely dissolve at 37°C. For the loading assays, several sets of DNA films based on PAMAM dendrimers (G3, G4 and G5) or brPEI25, at a N/P ratio of 4, were prepared in 2.0 mL SC tubes using the previously optimized experimental conditions. After obtaining the films (always working in the vertical laminar flow chamber), their supernatants were removed for free DNA quantification and the films were rinsed with 1mL of PBS. Afterwards, the DNA films were exposed to different concentrations of drugs (1 mL, 0 - 300µM for DOX, 0 - 600 µM for cisPt, and ≈ 384 µM for 5-FU), for different incubation times (24 h, 48 h and 72 h). After each time point, different procedures were followed according to the drug quantification protocol. For DOX quantification, 5 µL of the supernatants were withdrawn from the

DOX-incubated DNA films and made up to 100 μL with PBS solution in the wells of an opaque 96-well plate. Doxorubicin fluorescence was measured in the microplate reader, with $\lambda_{\text{ex}} = 530 \text{ nm}$ and $\lambda_{\text{em}} = 590 \text{ nm}$. Due to the low molar absorptivity of cisPt in the UV region, its quantification was done based on a derivatization reaction following a protocol adapted from Basotra *et al.*⁵⁴⁹. Briefly, 100 μL of the supernatant was withdrawn from the cisPt-incubated DNA films and transferred to 1.5 mL microcentrifuge tubes. After, 100 μL of OPDA (1.4 mg/mL in DMF) and 200 μL of PBS solution (pH = 6.8) were added and the solution was mixed by vortexing. The tubes were then heated in the thermoblock at 99°C, for 15 min. After cooling down, 600 μL of DMF was also added and the solution was mixed again. The quantification of free cisPt was determined by UV-vis spectrophotometry (absorbance at 706 nm). For 5-FU quantification, 300 μL of the supernatant was withdrawn from the 5-FU-incubated DNA films and made up to 1000 μL with PBS solution. Absorbance was then measured at 266 nm. Standard calibration curves for each drug were prepared in the same conditions used for the samples. The percentage of loaded drug was calculated using the following equation:

$$\% \text{ loaded drug} = \frac{\text{Drug}_{\text{free}}}{\text{Drug}_{\text{to}}} \times 100 \% \quad (4)$$

where $\text{Drug}_{\text{free}}$ corresponds to the drug concentration present in the supernatant (μM) and Drug_{to} the initial drug concentration in the incubation solution. Results are presented as mean $\pm$ s.d., with $n = 5$.

2.7.2. Drug release profiles

The DOX and cisPt release profiles from the DNA films were studied in conditions mimicking the physiological environment. To attain this objective, several sets of DNA films (based on G3, G4, G5 or brPEI25, at a N/P ratio of 4) were prepared, washed with 1 mL of PBS and incubated, separately, with 50 μg of DOX (92 μM) and 50 μg of cisPt (166 μM), in 1 mL of PBS solution, for 72 h. Afterwards, the supernatant was removed (free DNA and drug were quantified in the supernatant, as previously described), the films were rinsed with 1 mL of DNase/RNase free H_2O and, then, incubated with 1 mL of release solution. Studies were made in different release solutions, namely: DNase/RNase-free H_2O , 1x PBS (pH = 7.4); DMEM (pH = 7.4); 1% FBS (in 1x PBS), 10% FBS (10% in 1x PBS) and complete DMEM (DMEM with 1% AA and 10% FBS). At different time points, 25 μL and 100 μL of the incubation solution were removed, respectively, from DOX and cisPt incubated DNA films for quantification (as previously described) and replaced with the same volume of fresh incubation

solution (always working in the vertical laminar flow chamber). The cumulative release (%) was calculated using the following equation:

$$\text{Cumulative release (\%)} = \frac{V_t}{V_0} \times R_{t-1} + R_t \quad (5)$$

where V_t corresponds to the withdrawn volume (0.025 mL and 0.1 mL for DOX and cisPt respectively), V_0 to the volume of the incubation solution (1 mL), R_t is the release obtained at the actual time point (in %) and R_{t-1} is the release obtained in the previous time point (in %). The calculations take into account the initial amount of drug loaded into each film. Results are presented as mean $\pm$ s.d., with $n = 5$.

2.7.3. Cytotoxicity studies using cancer cell lines

For starting, DOX and cisPt half maximal inhibitory concentrations (IC_{50}) were determined after 24 h, 48 h and 72 h of exposure to ovarian cancer cells. A2780 and A2780cisR (latter resistant to cisPt) were cultured until 60%-80% confluence in cell culture dishes, using RPMI 1640 cell culture medium, supplemented with 10% FBS, 1% AA and 2 mM L-Glut. For A2780cisR cells (as recommended by the supplier), the medium was supplemented with 1 μ M cisPt every time a passage was made. Cells were incubated at 37°C, in a humidified atmosphere containing 5% CO_2 . When cells reached confluency, they were washed with pre-warmed PBS solution and were trypsinized (using trypsin-EDTA for 5 min in the incubator). After, the cell culture medium was added and cell density was determined. Cells were then seeded in 96-well plates at a density of 5×10^3 cells/well, in a final volume of 100 μ L of complete medium. Twenty four hours post-seeding, the medium was replaced by fresh complete culture medium (without cisPt, in the case of A2780cisR) with 10% (v/v) of a drug stock solution prepared in PBS. Cells were exposed to several final drug concentrations, namely, 0 - 0.5 μ M DOX and 0 - 10 μ M cisPt (A2780) or 0 - 1 μ M DOX and 0 - 100 μ M cisPt (A2780cisR), for 24 h, 48 h and 72 h. After each time point, cytotoxicity was evaluated using the resazurin reduction assay, as previously described. The IC_{50} values were calculated with GraphPad Prism 7.03, with a non-linear fit of inhibitor concentration vs. normalized response.

A2780 and A2780cisR cells were exposed to drug-loaded DNA films using transwells. For these experiments, DNA films were prepared based on G5 PAMAM dendrimers and PEI, at a N/P ratio of 4. Films were prepared in 2 mL SC tubes (as previously explained), rinsed with DNase/RNase-free H_2O and incubated for 72 h with 1 mL of 50 μ g/mL of DOX, 1 mL of 50 μ g/mL of cisPt and 1 mL of a solution containing both 50 μ g of DOX and 50 μ g of cisPt. Afterwards, the

supernatants were removed (free DNA and drugs were quantified), the films were washed with PBS solution and lyophilized for 3 days. Twenty four hours prior to film exposure, transwells were placed in 24-well cell culture plates and cells were cultured inside these transwells according to the supplier's instructions, at a density of 5000 cell/transwell, with 200 μ L and 1 mL of complete medium (without cisPt, in the case of A2780cisR) in the upper and lower compartments, respectively. In the following day, the cell culture medium was removed, and the lyophilized films were removed from the tubes and placed on the bottom of the wells (lower compartments). Fresh complete medium was added to the wells (400 μ L and 1 mL, for upper and lower compartments, respectively). Cells were incubated in these conditions for 72 h, after which the resazurin assay was performed. Resorufin fluorescence was measured in 100 μ L of medium. Results are presented as mean $\pm$ s.d., with n = 3.

2.8. Statistics

All statistical analysis were performed using GraphPad Prism 7.00. Ordinary one-way ANOVA with Tukey test was used to assess the statistical differences between group means. Results are reported as mean $\pm$ standard deviation.

3. Results and discussion

Poly(amidoamine) (PAMAM) dendrimers have been widely studied as gene (plasmid DNA, pDNA)^{520,550}, small interfering RNA (siRNA)^{500,551} and antisense oligonucleotide (ASO)^{552–555} carriers having in view gene therapy applications⁵⁰⁶. In fact, since they present a very high number of amine groups at their surface that are protonated at the physiological pH, they can easily establish electrostatic interactions with negatively charged nucleic acid molecules. When PAMAM dendrimers and nucleic acids interact together forming a complex (dendriplex), the negative charge of the nucleic acids is neutralized and, simultaneously, DNA suffers a compaction process⁵⁵⁶. Both these events will favour the entry of the nucleic acid inside cells and increase the success of transfection⁵⁵⁷. DNA is also compacted inside the nucleus of eukaryotic cells forming the chromatin⁵⁵⁸. The structure of chromatin is still nowadays poorly understood despite the extensive research in the field. However, it is known that it is made of basic repeated elements called nucleosomes that are interconnected by linker DNA. The nucleosome contains special condensing proteins, designated histones, around which the DNA wraps. These histones are highly basic proteins that establish with DNA several types of interactions (e.g. electrostatic attraction, hydrogen bonds, non-polar interactions) that together surpass the energy barriers for compaction. In a nucleosome, about 147 base pairs (bp) of DNA are wrapped around a group of eight positively charged histones (histone octamers) to form a nucleosome core particle. Linear arrays of nucleosomes are then formed by the linker DNA, giving rise to a structure called “beads-on-a-string” (Figure 62). The use of dendrimers as a model for DNA compaction by histones is based on their geometrical similarity with histones, although interactions between DNA and histones go beyond electrostatics^{559–561}, such as sequence specificity. Indeed, generation 6 PAMAM dendrimers, which have a diameter of 6.7 nm, possess dimensions close to those in histones. In addition, when one-third of their amine surface groups are protonated, they also show a positive charge near to that of histones^{562–564}.

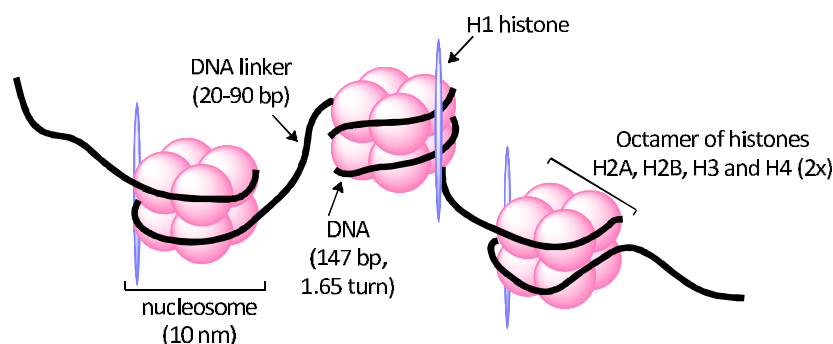


Figure 62 - Chromatine DNA compaction in eukaryotic cells. The primary structure of the chromatin is the nucleosome, composed by a histone octamer, a H1 histone and DNA linker. The linear array of nucleosomes with the DNA linker in between forms the beads-on-a-string structure.

Clearly, PAMAM dendrimers and DNA strongly interact with each other, thus revealing the possibility of creating a myriad of new materials with interesting technological and biomedical applications. Having this in view, in the beginning, we aimed at developing novel hydrogels based on DNA and dendrimers using a strategy that was never reported in the literature. The idea was to use long DNA molecules and to submit them to a temperature higher than their T_m , so that ssDNA could be obtained. Then, maintaining the ssDNA molecules separated at a high temperature, and before letting the system cool down, to put the dendrimers in contact with the DNA. By doing this, we expected to produce 3D hydrogels based on DNA where dendrimers (through electrostatic interactions) and random nucleobase pairing could generate a crosslinked structure capable of retaining water molecules. Interestingly, when the process was followed by slow cooling, a soft film appeared in the bottom of the tube. In fact, instead of 3D hydrogels, 2D films were developed that represent a new class of DNA/dendrimer materials.

3.1. Optimization process for SS-DNA/dendrimer films preparation

An important part of the work of this thesis corresponded to the optimization of the experimental conditions used to prepare the DNA/dendrimer films, simultaneously evaluating the impact on film formation of different parameters, such as the maximum temperature applied (for DNA melting), the cooling programme, the solution ionic composition, the solution's initial pH, the N/P ratio, the SS-DNA concentration, the PAMAM dendrimer generation, and the SS-DNA size and architecture. Figure 63 exemplifies the optimized procedure for the preparation of a DNA film based on G3 PAMAM dendrimers at N/P ratio of 4 (the N/P ratio is the molar ratio between the number of amine groups in the dendrimers and the number of phosphate groups in the DNA).

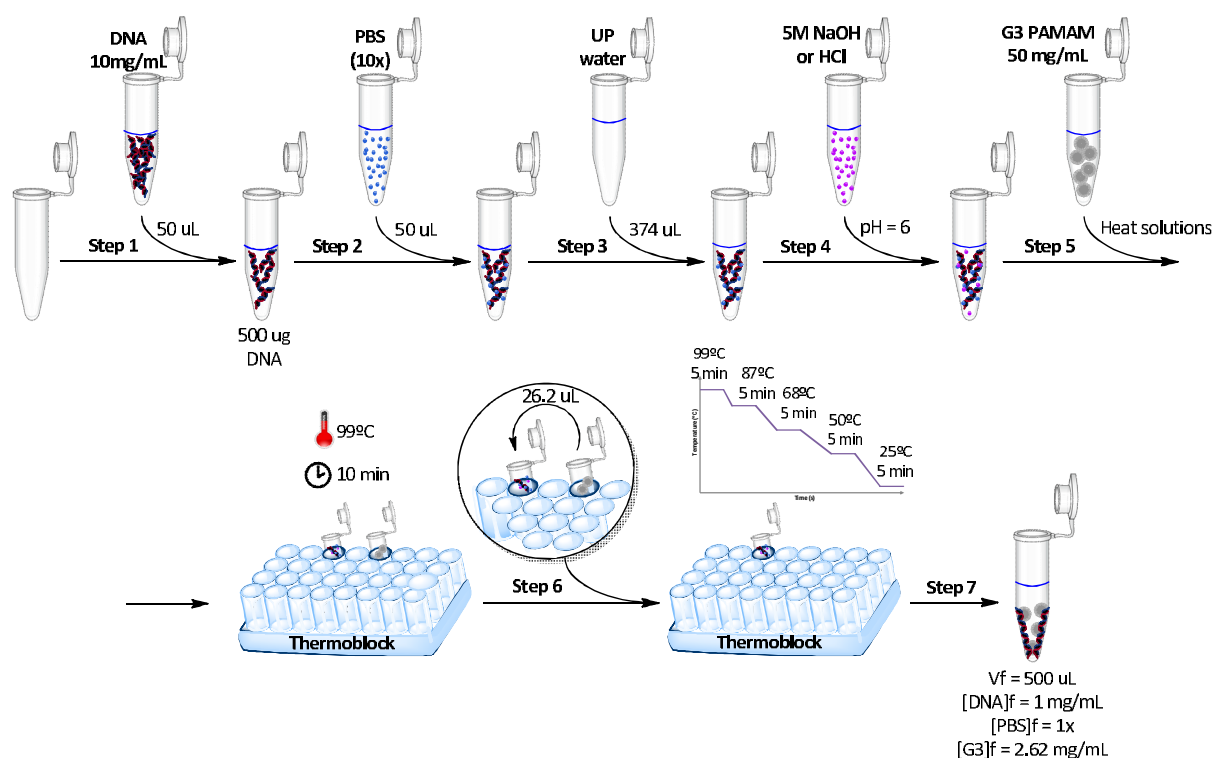


Figure 63 - Scheme of the optimized procedure and experimental conditions to prepare DNA/dendrimer films (G3 PAMAM dendrimer, N/P ratio 4).

Maximum temperature applied

Temperature is a parameter that strongly affects nucleic acid structure. Particularly, when double stranded DNA (dsDNA) is heated, it denature, that is, the double strand separated into two single strands²¹. By definition, the melting temperature (T_m) of a DNA molecule is the temperature at which half of its strands are denaturated, that is, exist 50% as ssDNA and 50% as dsDNA. The strategy followed to prepare the DNA/dendrimer films included an initial DNA heating step to separate the SS-DNA into two single strands. To optimize film preparation and, in addition, to understand the importance of the maximum temperature initially applied to the SS-DNA solution (in PBS) in the process, several attempts to prepare DNA/dendrimer films were done using G5 PAMAM dendrimers and heating at different temperatures, namely 25°C, 50°C, 68°C, 87°C and 99°C. Cooling was always performed using a controlled temperature ramp (CTR). The formation of DNA/dendrimer films was only observed when the SS-DNA was heated to 99°C, a temperature higher than its T_m (87°C). Heating at lower temperatures did not result in film formation. In fact, when the solutions containing the PAMAM dendrimers were added to the solutions containing the SS-DNA (followed by immediate mixing with a micropipette), a non-homogeneous soft aggregate that looked like a spiderweb (or a clot) was formed in solution, near the micropipette tip, sometimes ending up by clogging the tip. As the solution was homogenized, the clot turned to be denser. Figure 64 shows the appearance of the

“spiderwebs” upon addition of G5 PAMAM dendrimers to a SS-DNA solution, at the room temperature of 25°C (these experiments were done for several N/P ratios). The formation of similar clots was observed for the other tested temperature values. This may be related to the kinetics of neutralization, since with fully denatured DNA, the dendrimers are able to quickly condense ssDNA, which has half of the charges of dsDNA. Films were only produced when the starting SS-DNA and dendrimer solutions were heated until 99°C. The interaction of PAMAM dendrimer with ssDNA and dsDNA is known to be different. While its flexibility is different (ssDNA > dsDNA), ssDNA penetrates more deeply into the PAMAM dendrimer branches. It must be noticed that dendrimers' solutions were always also heated to avoid a huge temperature drop in the system upon addition to the SS-DNA solutions. PAMAM dendrimers are not affected by the increased temperature as, according to the manufacturer⁵⁶⁵, it only decomposes at 150°C. In conclusion, the maximum temperature initially applied to the SS-DNA solution was crucial to achieve DNA film production, thus revealing the importance of the extent of DNA melting in the process.

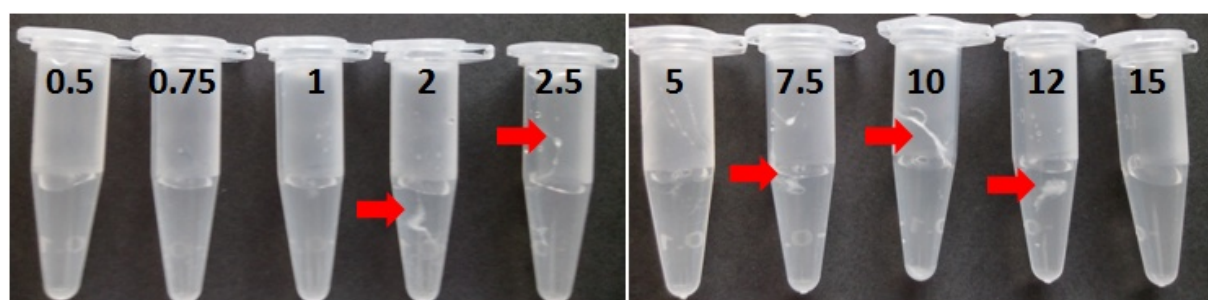


Figure 64 - Maximum heating temperature influence on DNA denaturation and film preparation. Films are not formed upon addition of G5 PAMAM dendrimers to SS-DNA solutions at room temperature (these experiments were done at several N/P ratios, from 0.5 until 15). Red arrows indicate the “spiderwebs” formed.

Cooling programme

The cooling process had an important role in film formation too. In this context, three cooling programmes were tested: on ice (0°C, disables hybridization), at room temperature (RT, known to induce hybridization of small DNA fragments) and using a controlled temperature ramp (CTR, 99°C to 87°C, 68°C, 50°C and 24°C; keeping the temperature value constant for 5 minutes at the referred temperature points). The choice for a slow controlled cooling process like CTR was, partially, inspired by the Polymerase Chain Reaction (PCR) technique. To evaluate the importance of the cooling process in film formation, G5 PAMAM dendrimers were again used. This optimization study was done simultaneously with the analysis of the influence of other experimental parameters, such as the solution ionic composition and the N/P ratio. After all experiments, stable film formation was only

observed when the cooling occurred using CTR conditions, for N/P ratios in the range 3 – 6, and when SS-DNA was dissolved in PBS (Figure 66). In conclusion, the methodology selected for cooling was determinant and slow cooling, like the one achieved using CTR conditions, was necessary to attain the objective of film formation. From a thermodynamic point of view, a slow decrease in temperature means that the system has a longer time to reorganize which, in this case, means more time and adequate temperature for complete hybridization¹²⁵ and, possibly, to adopt other kind of ordered structure. For example, it is reported that small DNA strands can form liquid-crystalline spherulites from solutions upon slow cooling⁵⁶⁶. In addition, it is well known, in the materials science field, that polymers may form semicrystalline structures⁵⁶⁷.

Knowing that the films were only formed if a slow cooling was applied after heating at 99°C, the effect of the cooling process on SS-DNA hybridization was investigated by agarose gel electrophoresis retardation assays. As can be observed in Figure 65, SS-DNA was mainly composed of molecules with approximately 12 kbp, although smearing was present revealing the presence of DNA fragments with lower sizes (lane 2). After the heating and cooling process on ice (lane 3) or RT (lane 4), the strong SS-DNA band disappeared, even if some smearing was still visible along the run, with a slightly higher intensity near the SS-DNA mark. SS-DNA suffered denaturation after the heating step. Upon a fast cooling step (on ice or at RT) hybridization was not achieved, thus DNA remained as single stranded. As such, ethidium bromide that is used as a label to detect dsDNA in agarose gel assays (by intercalating between DNA base pairs) did not give a signal^{568–570}. The results, however, were quite different for the SS-DNA submitted to CTR (lane 5). The band at 12 kbp disappeared but a strong signal from SS-DNA was present in the well, indicating that SS-DNA's size increased, thus being retained in the well. This result shows that the long SS-DNA passed through a hybridization process that, as expected, due to the size of the molecules, was not perfect (random hybridization) and gave rise to a crosslinked structure. In fact, the probability of a long DNA strand to fully hybridize with only one other strand is very low²³. The result was the retention of the "crosslinked" DNA in the well. So, this assay clearly showed that the application of a CTR, through slow cooling, enabled a certain degree of hybridization in the SS-DNA, that is, gave time to the system to self-organize.

A very interesting revelation came from these agarose electrophoresis experiments by introduction of G5 PAMAM dendrimers in the system. When the SS-DNA was not heated and was mixed with the dendrimers, no signal was detected in the gel (lane 6). The same happened when the SS-DNA was heated and cooled, independently of the cooling process used (fast or slow) (lanes 7 - 9). We can only attribute this result to the impossibility of labelling the DNA with ethidium bromide (EtBr) due to a very efficient DNA compaction⁵⁷¹. Dendrimers are, then, capable of condensing both ssDNA and dsDNA in a very intense manner. In conclusion, under the experimental conditions of film

formation, SS-DNA should only suffer a partial hybridization but both single and double strands are compacted by dendrimers.

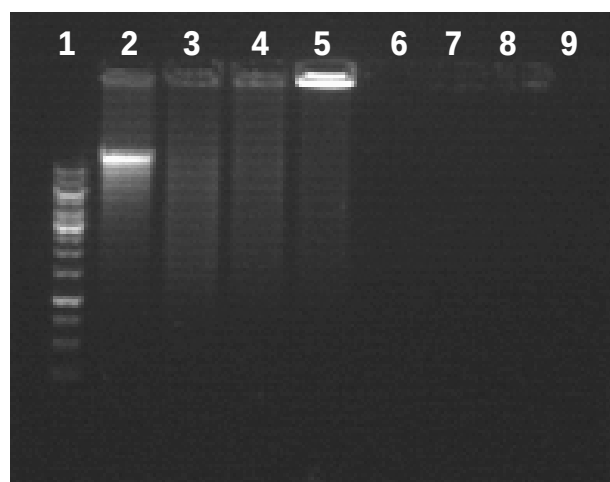


Figure 65 - Agarose gel electrophoresis retardation assay of SS-DNA in PBS. Lanes correspond to: GeneRuler 1kb DNA ruler (1); SS-DNA (not heated) (2); SS-DNA heated and cooled in ice (3); SS-DNA heated and cooled at RT (4); SS-DNA heated and cooled using a CTR (5); SS-DNA (not heated) + G5 PAMAM (N/P = 4) (6); SS-DNA heated + G5 PAMAM (N/P = 4, cooled in ice) (7); SS-DNA heated + G5 PAMAM (N/P = 4, cooled at RT) (8); and SS-DNA heated + G5 PAMAM (N/P = 4, cooled with CTR) (9).

Ionic composition of the solution

Attempts to prepare DNA/dendrimer films were made using media with different ionic compositions, namely UP H₂O, 0.15 M NaCl, Tris-EDTA (TE) buffer, and PBS solution. Figure 66 shows the appearance of the solutions obtained during these assays using G5 PAMAM dendrimers. When CTR cooling was applied, films were formed but only in PBS solution. In water, TE buffer and in 0.15 M NaCl, the solutions became turbid with some of them containing suspended aggregates. After repeating this study several times, also using G3 and G4 PAMAM dendrimers, the same result was always obtained, that is, in PBS solution, after touching the bottom of the microcentrifuge tubes with a micropipette tip, a soft, very thin film could be removed. These films presented a relatively high resistance. PBS solution is a common buffer used in biological experiments that has NaCl, KCl, KH₂PO₄ and Na₂HPO₄ in its composition. When comparing with water, for example, PBS presents a higher ionic strength, a parameter that influences the electrostatic interactions between SS-DNA and dendrimers in solution. As a matter of fact, Su *et al.*⁵⁷² reported that increasing salt concentration resulted in less DNA interaction and condensation (as Na⁺ ions shield the phosphate groups), which may be the reason behind the fact that DNA films were not obtained in solutions with a lower ionic strength, such as in water and TE buffer, due to higher DNA/dendrimer interaction and tighter DNA condensation. In other words, less condensation at higher ionic strength (*i.e.* PBS) may allow the presence of free ssDNA strands for posterior hybridization and thus, forming the film. Also, it is known that the ionic strength affects DNA duplex stability^{11,573}. However, PBS and NaCl solutions

have similar ionic strengths (≈ 0.15 M) and the films are only formed in the first solution. This finding points out the importance of the solution composition in the process.

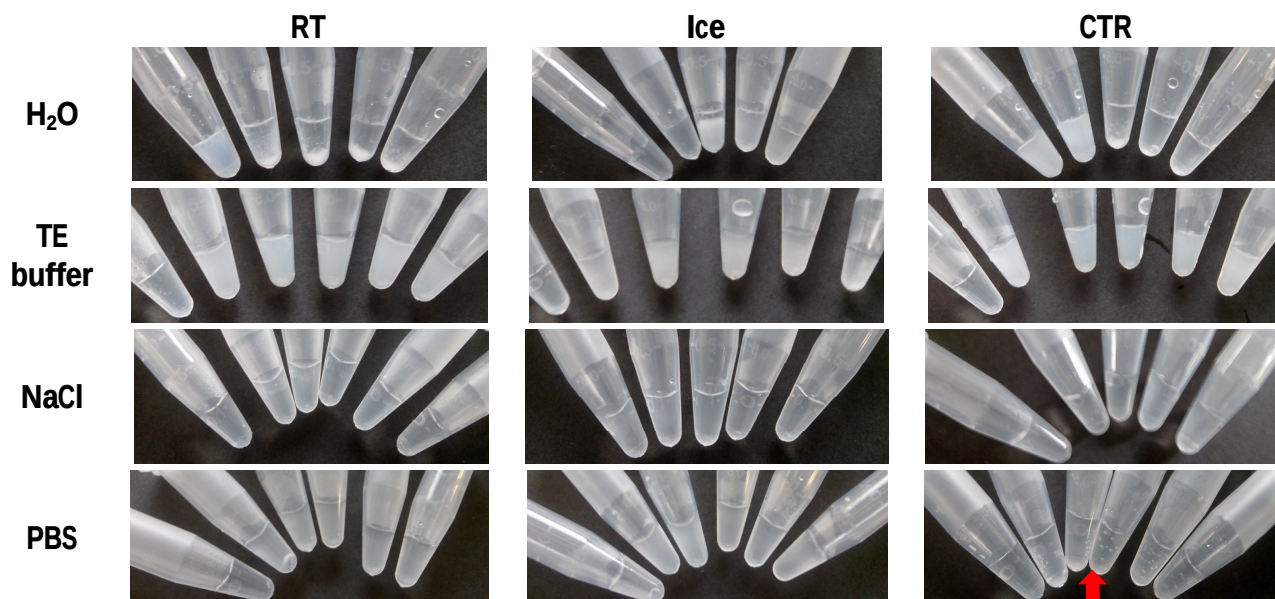


Figure 66 - Attempts to prepare DNA/G5 PAMAM dendrimer films using different cooling processes, solutions and N/P ratios. Initial DNA solution prepared in H₂O (N/P ratio 2, 2.5, 5, 7.5 and 10, in this order), TE buffer (N/P ratio 0, 1, 5, 7.5, 10 and 15, in this order), 0.15 M NaCl (N/P ratio 1, 5, 7.5, 10 and 15, in this order), and PBS solution (N/P ratio 0, 1, 5, 7.5, 10 and 15, in this order), followed by heating at 99°C and cooling at RT, on ice and following a CTR. Film formation was only observed in condition PBS-CTR, on the bottom of the microcentrifuge tubes.

In fact, we also found out that when PBS solution containing magnesium ions is used (0.5 mM MgCl₂, like that used in cell culture experiments), the film is not obtained. Actually, it is known from the literature that divalent magnesium ions (Mg²⁺) are able to interact with DNA's backbone phosphate groups, shielding DNA's negative charge and increasing duplex's stability¹⁸ (resulting in an increase of the DNA's T_m)¹¹. It is reported that binding of Mg²⁺ to DNA must be considered when both Mg²⁺ and phosphate group concentrations are of similar order of magnitude¹⁸, which happens in this case (0.5 mM Mg²⁺ and 3.03 mM DNA phosphate). Furthermore, magnesium ions can bind to both dsDNA and ssDNA. The binding of Mg²⁺ to denatured SS-DNA may influence the interactions between DNA and other molecules such as cationic polymers, as it may compete with the polymers for the interactions with DNA's phosphate groups⁵⁷⁴. Summarizing, the impossibility of obtaining DNA/dendrimer films in the presence of magnesium ions may find an explanation in just one or in the combination of these two reasons – that is, an increase of the T_m or/and a diminishment of the electrostatic attraction between DNA and dendrimers. To better understand what was happening, agarose gel electrophoresis retention assays were also done to compare the hybridization behaviour of the SS-DNA in the presence of magnesium ions. As can be seen in Figure 67, there were no differences between SS-DNA migration in the agarose gel when using PBS solution with or without

Mg²⁺ ions. The results regarding the position of the bands in the agarose gel were already previously discussed. Thus, it seems that the possible increase in T_m promoted by the presence of these ions in the system was not sufficient to impair the melting of DNA when 99°C were applied.

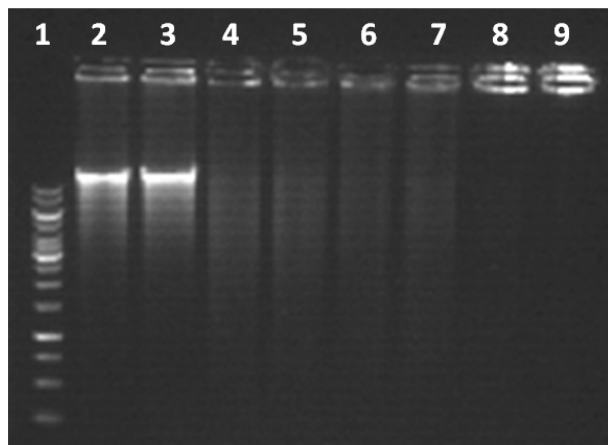


Figure 67 - Agarose gel electrophoresis retardation assay of SS-DNA. Lanes correspond to: GeneRuler 1kb DNA ruler (1); SS-DNA (not heated) in PBS (2) and in PBS-Mg²⁺ (3); SS-DNA after heating and cooling in ice in PBS (4) and PBS-Mg²⁺ (5); SS-DNA after heating and cooling at RT in PBS (6) and PBS-Mg²⁺ (7); SS-DNA after heating and cooling by a CTR in PBS (8) and PBS-Mg²⁺ (9).

A study by Conwell *et al.* showed that magnesium ions interfered in DNA condensation promoted by hexamine cobalts (III)⁵⁷⁴. In our case, since magnesium ions did not had an important impact in SS-DNA itself and in its hybridization behaviour, it was decided to investigate deeper their possible influence on the electrostatic interactions established with the dendrimers. For this study, the Picogreen® assay (a method frequently used to quantify dsDNA down to the picogram range) was applied to evaluate SS-DNA condensation. This assay is based on a fluorescent intercalator dye – the Picogreen reagent – which fluorescence is triggered by binding to free dsDNA (it can bind to ssDNA but in a negligible extension). As DNA is condensed by a polymer, the sites for the dye intercalation are affected and the fluorescence intensity decreases. So, this assay was done by preparing SS-DNA/G5 PAMAM dendriplexes in PBS, with and without magnesium ions (PBS-Mg²⁺ and PBS), in two conditions, without heating the DNA and following the heating and CTR cooling process. Results are summarized in Figure 68. From the plotted graphics, one can conclude that the presence of magnesium ions does not significantly affect the condensation behaviour of SS-DNA. The Picogreen's supplier mentions that MgCl₂ up to 50 mM may interfere with the assay, decreasing the fluorescence by 33%. In these experimental conditions, that was not verified, as MgCl₂ was at a concentration 100-fold lower (0.5 mM). As can be seen, when the N/P ratio is 0, the fluorescence intensity values are similar for both PBS-Mg²⁺ and PBS. When dendrimers are added to the system, DNA compaction occurs and increases with the N/P ratio. After achieving a critical N/P ratio (in this case, at N/P ratio 1), compaction attains a maximum value and the dendrimers contribute for providing a higher positive charge to the formed complexes. Dendrimers in excess can also remain in solution.

Interestingly, at a N/P ratio of 0 (no dendrimers added to the system), the fluorescence signal is much lower after the heating and cooling process. This indicates that after DNA denaturation and hybridization (without dendrimers), the amount of dsDNA decreases and, possibly, due to the random crosslinking occurring during hybridization (observation supported by the agarose gel electrophoresis retardation assay, Figure 65), the dye may also have more difficulties to penetrate in the produced mesh. Since Picogreen® needs to intercalate in dsDNA to be fluorescent, fluorescence intensity thus presents lower values.

Although the agarose gel electrophoresis assays and the Picogreen® assay could not be done exactly at the same SS-DNA concentrations used to prepare the DNA/dendrimer films (lower concentrations were used), overall, they seem to point out that magnesium ions do not interfere with the SS-DNA hybridization process after heating and cooling, as well as with its electrostatic interactions with dendrimers. So, despite the efforts made, no explanation was found for magnesium ions interference in film formation. Nevertheless, from this point forward, the use of PBS without magnesium ions was established as essential for film fabrication.

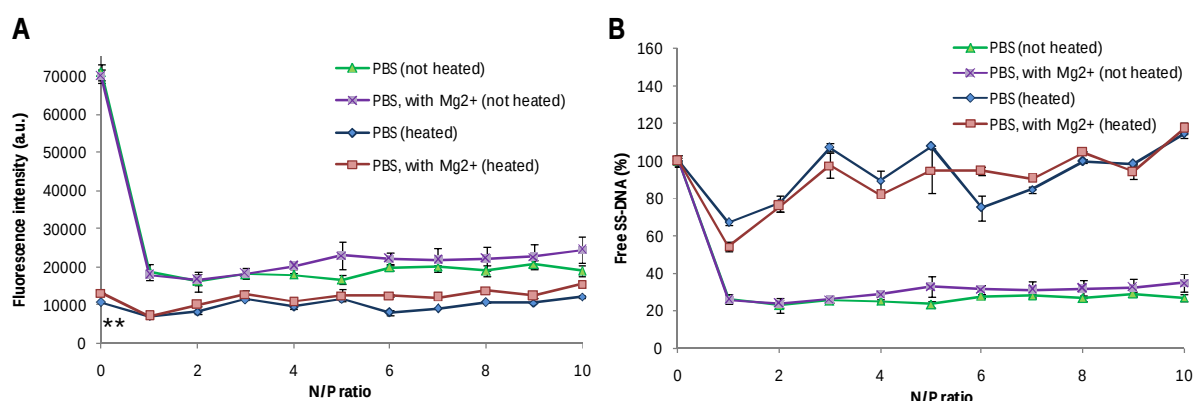


Figure 68 - SS-DNA condensation carried out by G5 PAMAM dendrimers (at N/P ratios from 0 to 10) in PBS and PBS-Mg²⁺. Experiments were done without heating the DNA, and heating it and then cooling it down using a CTR. (A) fluorescence intensity and (B) percentage of free SS-DNA in relation to N/P ratio 0. Results are expressed as mean $\pm$ s.d., with n = 3.

SS-DNA solution's initial pH and N/P ratio

As mentioned before, the degree of protonation (and thus, surface charge) of the dendrimers is strongly affected by the pH. As such, during the optimization process, the pH of the initial SS-DNA solution was also a studied parameter. The 10x PBS stock solution used for film preparation has an initial pH value of 6.8. When 10x PBS is diluted to 1x PBS with H₂O, the pH becomes approximately 7.4. However, the pH value changes for 6.95 ± 0.10 when SS-DNA is present, due to the acidic character of DNA. The influence of the SS-DNA solution's initial pH on film formation was then

evaluated by adjusting pH values to 5, 6, 7 and 8 (± 0.05) prior to the heating step. Attempts to prepare films used G5 PAMAM dendrimers at different N/P ratios. The pH values attained at the end of the process (final pH) were registered, as well as conditions under which films were obtained. Distinct responses were observed in these experiments which can be seen in Table 2 and Figure 69. First, one should notice that, independently of the initial pH, the final pH values are always higher, reflecting the addition of a base (dendrimer) in an acid (SS-DNA) solution. Also, maintaining constant the initial pH, the final pH values always increased with increasing N/P ratio since the amount of basic species (amine groups) in solution increases too.

Despite the similarity observed in the behaviour of the systems in relation to pH variation, the films did not form in all situations. Indeed, stable films were only achieved when the initial pH of the solution was adjusted to 6. These results point out that the level of dendrimer protonation strongly affects film formation. As stated before, PAMAM dendrimers have internal tertiary amine groups with a pKa around 4 and surface primary amine groups with a pKa around 7^{575,576}. Thus, primary amines have higher affinity for protons in alkaline environment and tertiary amines in acidic pHs (protonation occurs in a “core-shell” manner – first in the periphery and, after, inside dendrimer cavities)⁵⁷⁵. In fact, this behaviour makes PAMAM dendrimers good vehicles for nucleic acid delivery inside cells as they present a high buffering effect inside lysosomes (they act like “proton sponges”⁵⁷⁷), protecting the nucleic acid from acidic degradation. The protonation behaviour of PAMAM dendrimers is, so, dominated by the chemical environment of the involving solution^{575,576}.

The results obtained clearly confirm that stable film formation is strongly dependent on the electrostatic interactions established between dendrimers and SS-DNA. On one hand, dendrimers must be strongly protonated, which only occurs for pHs less than 7⁵⁷⁶, on the other hand SS-DNA must keep its negative charges which tend to decrease if the pH value starts to be too much acid²⁶¹. This can explain why stable film production only takes place at pHs between 6 and 7, since the dendrimers are protonated. A possible explanation for the impediment of film formation at a pH of 5 is that dendrimers' charge is so high, and the electrostatic interactions between dendrimers and SS-DNA are so intense, that the SS-DNA strands with unpaired bases do not have enough flexibility to move and hybridize with neighbor strands⁵⁷². This is, of course, based on the hypothesis that film formation depends on the electrostatic interactions between DNA and dendrimers but also on base pairing among DNA strands denatured after the heating process. The results in Table 2 and Figure 69 also showed that, for an initial pH of 6, stable film formation only occurred at a N/P ratio of 5 (for the higher N/P ratios 7.5 and 10, very fragile films were prepared that could not be removed from the bottom of the tubes without being damaged). Indeed, further experiments done under optimized experimental conditions, revealed that it was possible to obtain stable films for a N/P ratio ranging from 3 to 5.

Table 2 - Effect of the pH of the initial SS-DNA solution on film formation. Experiments were done at different N/P ratios and using G5 PAMAM dendrimer. Results are represented as mean, with n = 3.

		N/P ratio					
		0	1	2.5	5	7.5	10
pH 5	Final pH	5.6	6.7	7.7	8.7	9.0	9.2
	Appearance	Clear	*	*	*	*	*
pH 6	Final pH	6.3	7.1	8.3	8.9	9.2	9.3
	Appearance	Clear	*	*	Film	Film (fragile)	Film (fragile)
pH 7	Final pH	7.3	9.0	9.3	9.5	9.6	9.6
	Appearance	Clear	*	*	*	*	*
pH 8	Final pH	8.3	9.3	9.5	9.6	9.7	9.7
	Appearance	Clear	*	*	*	*	*

* no film formation was observed

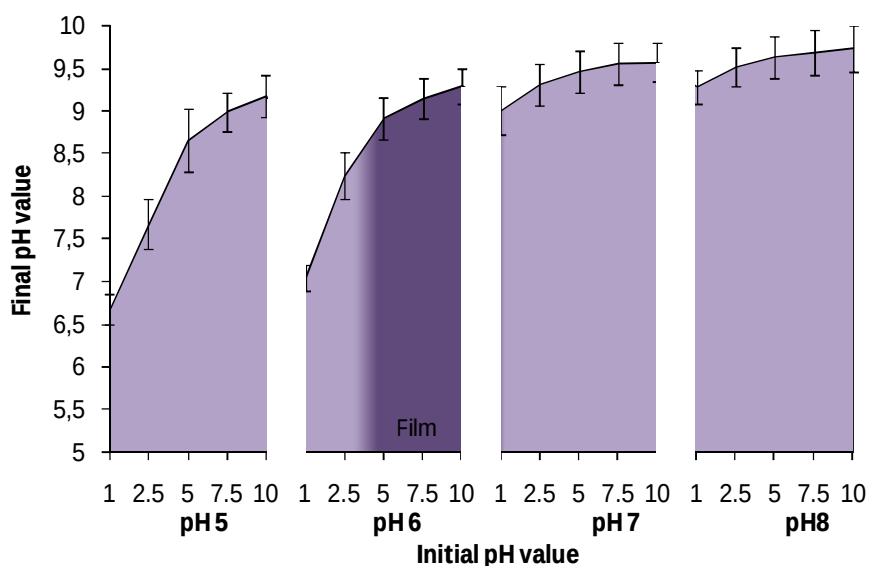


Figure 69 - Effect of the pH of the initial SS-DNA solution on the final pH achieved in solution and on film formation. Experiments were done at different N/P ratios and using G5 PAMAM dendrimer. Results are represented as mean $\pm$ s.d., with n = 3.

Although the previous results refer to attempts to produce films based on G5 PAMAM dendrimers at different N/P ratios, further experiments were also done to verify if the same behaviour was present when G3 and G4 PAMAM dendrimers were used. As can be observed in Figure 70, now fixing the SS-DNA solution' initial pH at 6, the evolution of pH values with increasing N/P ratios was very similar for the three dendrimer's generations. Importantly, stable films were formed for N/P ratios of 3 to 5 independently of the PAMAM generation used. So, it seems that after guaranteeing that dendrimers are protonated in the first moments of the process of film formation

by immersion in a solution with pH of 6, film formation is then essentially dependent on the charge ratio between the two components (N/P ratio), and not on the size of the dendrimer. This is very interesting, especially because it is known that dendrimers of different generation can form distinct types of structures when interacting with DNA⁵⁶⁰.

Taking all these results into account, a SS-DNA solution' initial pH of 6 and N/P ratios in the range 3 - 6 were selected to be used as optimal conditions for film formation.

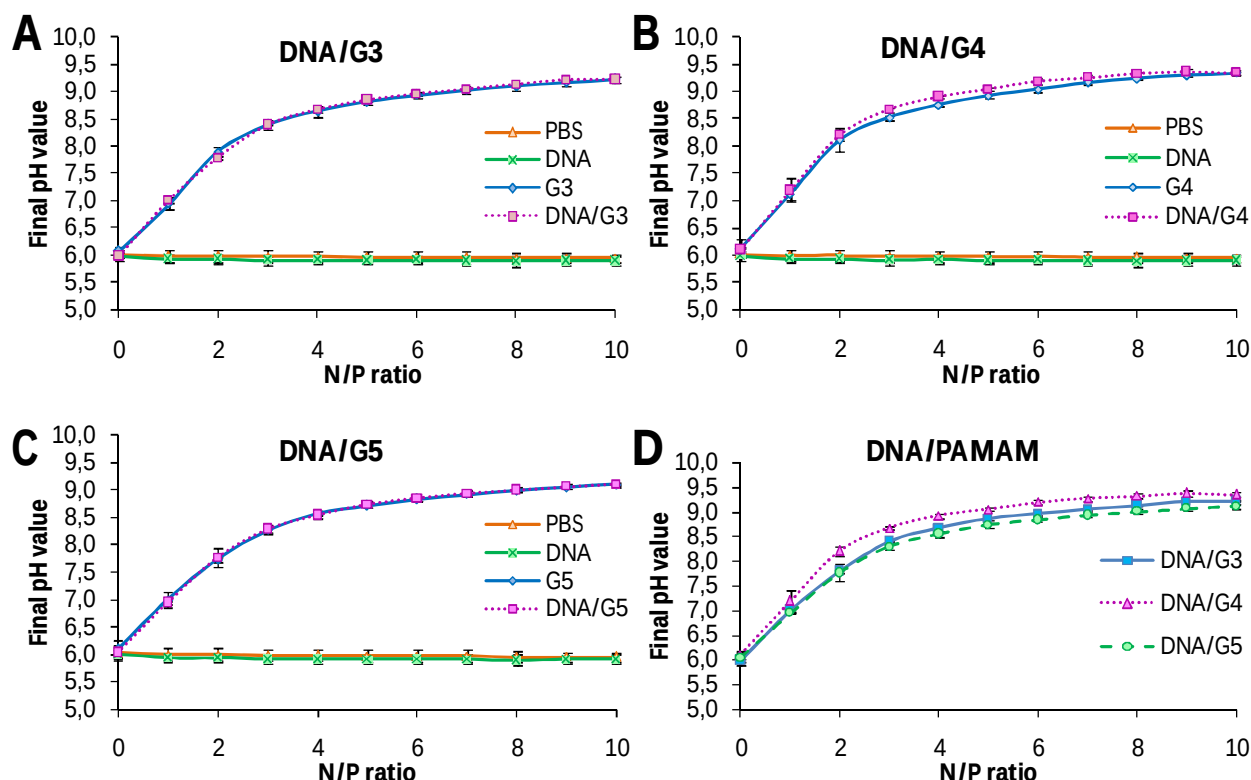


Figure 70 - Final pH achieved after addition of PAMAM dendrimers into SS-DNA solutions. Experiments were done fixing the SS-DNA solution' initial pH at 6 and using (A) G3, (B) G4 and (C) G5 PAMAM dendrimers. The pH behaviour obtained with the different dendrimer generations was compared (D). Results are represented as mean $\pm$ s.d., with n = 3.

Dendrimer generation

Despite what was said at the end of the previous section, it was possible to prepare DNA/dendrimer-based films using generations G3, G4 and G5 PAMAM dendrimers (higher generations were not tested due to their cost) but not using the lower generations (G1 and G2). A possible reason for this may be related with differences in dendrimer's shape which can condition their interaction with DNA. Indeed, it is described that while low dendrimer generations are quite planar structures, the higher generations tend to adopt a spherical shape⁵⁷⁸. As will be explained later in more detail, charge is much more concentrated in spherical molecules. This, for sure, will

influence the electrostatic interactions of dendrimers with DNA. There are several works describing the distinct behaviour of low and high generation dendrimers in the presence of DNA. For example, it was shown that, to condense the same amount of DNA, more molecules of low generation dendrimers are required when comparing with high generation dendrimers^{522,579}. Also, a study by Qamhieh *et al.*⁵⁷⁹ revealed that DNA wraps around the higher generation dendrimers due to their lower surface curvature. DNA wrapping is dependent of DNA's flexibility and dendrimer curvature. Also, the number of turns increases with the dendrimer size or generation. Nandy *et al.*⁵⁸⁰ and Su *et al.*⁵⁷², through molecular dynamics (MD) simulations, also showed that the driving force in DNA condensation carried out by dendrimers is based on electrostatic interactions and that higher dendrimer generations have bigger affinity towards DNA. Su *et al.*⁵⁷² also agreed that lower generation PAMAM dendrimers do not have enough charge density to condense one entire ssDNA molecule, producing unstable complexes, while the opposite happens with higher generations. Moreover, gene delivery studies using dendrimers show that the higher generations are much more efficient in DNA condensation and transfection⁵⁰⁶. In the context of the present work, the impossibility of using G1 and G2 PAMAM dendrimers to prepare films may have similar explanations. Their shape and very small size may impair their action as crosslinking agents among denaturated DNA strands and their low positive charge density is not enough to establish strong electrostatic interactions with DNA.

SS-DNA concentration

The impact of SS-DNA concentration on DNA/dendrimer film formation was also evaluated. Maintaining all other conditions constant, experiments were made changing the SS-DNA concentration in solution (0.2, 0.6, 1 and 2 mg/mL) and varying the N/P ratio. Interestingly, the range of N/P ratios necessary for film formation decreased with SS-DNA concentration. A series of experiments revealed that for low SS-DNA concentrations (0.2 or 0.6 mg/mL), films were formed in a N/P ratio range 7.5 to 10 (even if they were very thin and fragile). At a concentration of SS-DNA of 1 mg/mL, films formed in a N/P ratio range 3 to 5. At a concentration of SS-DNA of 2 mg/mL, films formed in a N/P ratio range of 2.5 to 5. These results are consistent with the idea that more dendrimers are needed to condense DNA molecules further away from each other in solution, as it is known that DNA condensation is a cooperative process, where free dendrimers would rather interact with already formed complexes than with free DNA molecules⁵⁶⁰. However, based on this possibility, it is expected that the films formed at different SS-DNA concentrations may have different internal organizations. As low DNA concentrations led to fragile films and high DNA concentrations do not

present advantages since more dendrimers have to be consumed in the process, a final SS-DNA concentration of 1 mg/mL was settled as standard.

SS-DNA size and shape

Attempts were made to create films using a plasmid DNA instead of linear SS-DNA. For these studies, a plasmid DNA (pDNA) encoding enhanced green fluorescent protein (EGFP) (pDNA-EGFP, 6.4 kb) was used. This pDNA is a circular DNA molecule and is approximately half the size (bp) of the SS-DNA. In the optimized conditions achieved for SS-DNA and using G5 PAMAM dendrimers (at a N/P ratio of 5), pDNA did not form a film. When adding the dendrimer, the solution became cloudy with formation of big flakes as the solution was homogenized. This may have happened due to the different molecular architecture and size of pDNA when comparing with SS-DNA. In addition, pDNA can degrade after heating at 100°C, as reported by Yan *et al.*⁵⁸¹. But, at the same time, studies conducted by Trovato *et al.*⁵⁸² showed that complete denaturation of circular DNA is forbidden due to strand interwinding, torsional stress and twisting. Due to these reasons, studies with pDNA-EGFP were not continued. Even if pDNA films were obtained, the amount of pDNA required would be very high. As such, considering the low quantity obtained in each DNA extraction and all the extracting reagents and time consumed, this process would not present huge advantages.

The effect of the size of the DNA strands in DNA/dendrimer film formation was also evaluated. For this purpose, SS-DNA was subjected to ultrasounds in a water bath. Ultrasounds promote the formation of cavitation bubbles and as the bubbles reach a critical size, they implode, releasing shearing forces that promote DNA shearing/fragmentation⁵⁸³. The usual procedure to obtain DNA/dendrimer films was followed using the sonicated SS-DNA. With great surprise, it was observed that it was not possible to obtain films starting with the sonicated SS-DNA (whether sonicated for a short or long time). Indeed, when adding the dendrimer, the solutions became very pale white and transparent (instead of white and turbid, as always happens when films are formed).

To determine what happened to SS-DNA after sonication, agarose gel electrophoresis retardation assays were performed (Figure 71). By observing the gel, it was possible to conclude that sonication resulted in a partial fragmentation of the SS-DNA as the characteristic band of native SS-DNA (not heated), at approximately 12 kbp, always appeared with a strong signal (lanes 4, 6, 8, 10, 12, 14 and 16). However, the intensity of this band was a function of the sonication time, revealing that some fragmentation occurred. Interestingly, this small extent in SS-DNA fragmentation was enough to impair film formation. We would, however, expect to observe the typical smearing or drag effect in the agarose gels upon ultrasound application⁵⁸⁴, corresponding to the presence of low molecular weight DNA fragments. The low DNA fragmentation achieved with the ultrasound bath is

certainly due to the ultrasound low intensity provided by this type of equipments. The intensity of these ultrasound baths is not comparable to immersion probe sonicators¹² or to other more advanced equipments. Also, the SS-DNA solution's high concentration may have influenced fragmentation, as viscous solutions influences the wave propagation, thus, the ultrasound efficacy⁵⁸³. But still, sonication has influenced SS-DNA to a point where no films were produced. Gel observation further allowed to conclude that the sonicated DNA, after being heated and cooled down through a CTR, suffered a hybridization process which prevented its migration, being retained in the well (lanes 5, 7, 9, 11, 13, 15 and 17). This behaviour was identical to the one previously observed for native SS-DNA and again confirmed in this experiment (lane 3). It seems, so, that although hybridization still occurs upon the heating and cooling steps, the effect of sonication over SS-DNA, leading to a partial (very little) fragmentation, prevents DNA/dendrimer formation.

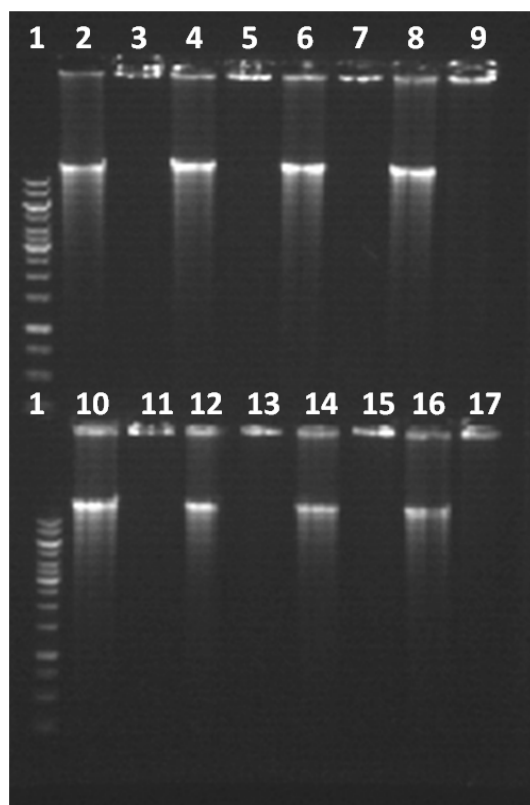


Figure 71 - Agarose gel electrophoresis assay of native and sonicated SS-DNA. Lanes correspond to: GeneRuler 1kb DNA ruler (1); native SS-DNA (2); native SS-DNA after heating and cooling using a CTR (3); SS-DNA sonicated for 10 min (4), 20 min (6), 30 min (8), 40 min (10), 50 min (12), 60 min (14) and 70 min (16); SS-DNA sonicated for 10 min (5), 20 min (7), 30 min (9), 40 min (11), 50 min (13), 60 min (15) and 70 min (17) after heating and cooling using a CTR.

3.2. Scale-up of the films

When DNA/dendrimer films are formed, they cover the bottom of the reservoir used for their preparation. The first experiments done in this work used 100 µg of SS-DNA in a total volume of 100 µL (after dendrimer addition). However, to better visualize and manipulate the DNA films, the

protocol was soon changed to 500 μg in a final volume of 500 μL , always using 1.5 mL conical microcentrifuge tubes. This already revealed that the developed system was able to be scale-up without the films losing stability. Images of DNA/dendrimer films (G5, N/P ratio 5) were captured using an inverted optical microscope (Figure 72), after removing them from the flask and placing them on the top of a glass coverslip. In general, films were almost transparent and homogeneous, seeming to have small particles homogeneously distributed at the surface (Figure 72, A). A few defects can be however observed in the images, namely some eruptions due to air bubble entrapment during the film preparation procedure (Figure 72, B). Because they are malleable and soft, care should be taken during their manipulation to avoid wrinkling, folding and ripping (Figure 72, C-D).

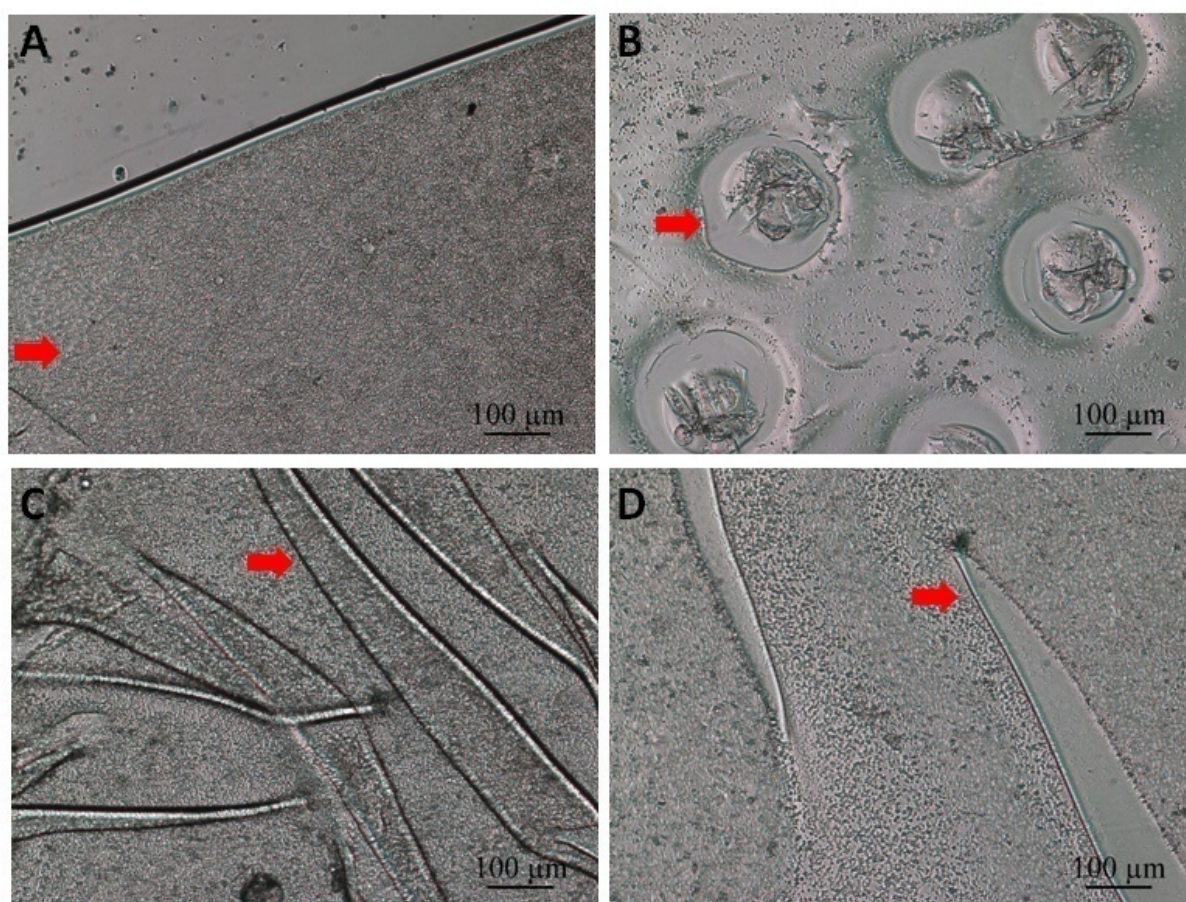


Figure 72 - Optical microscope images of a SS-DNA film based on G5 PAMAM dendrimers at a N/P ratio of 5, 100x magnification. Red arrows indicate (A) film (against the clear coverslip), (B) eruptions in the film, (C) wrinkling and folding of the film, (D) rips in the film.

The possibility of using other types of surfaces and containers, increasing even more the scale of the films, was further explored. For this purpose, other heating systems had to be used, such as incubators, in which it was not possible to programme a CTR. The approach was to heat the SS-

DNA solution at 100°C for 1 h. The heating time was extended from 10 min to 1 h, since the air inside the incubator needs to be heated first, contrary to what happens in the thermoblock (dry bath), where the microcentrifuge tubes are in direct contact with the heating plate. Lower heating times resulted in immediate precipitation and formation of wool-like clots. Glass vials of different size and with flat bottom were then used, such as 1.5 mL chromatography vials ($A = 1.06 \text{ cm}^2$), as well as other sample flasks ($A = 2.00, 3.76$ and 5.90 cm^2). Since the chromatography vials present a size similar to the conical microcentrifuge tubes previously used, 500 μg of SS-DNA in a final volume of 500 μL were also used for this case (films containing approximately $472 \mu\text{g}/\text{cm}^2$ are here produced). To maintain homogeneity among the films, this relation was always applied when using other flasks. So, for film preparation, SS-DNA and dendrimer (G5 PAMAM dendrimers at a N/P ratio of 5) solutions were heated in closed flasks (to prevent evaporation) inside an incubator, for 1h. After, the dendrimer solution was added to the SS-DNA solution. The addition and homogenization have been done as quickly as possible, to avoid big temperature drops which would result in precipitation and formation of wool-like clots. The solutions were cooled down, undisturbed, inside the incubators, after changing the set temperature from 100°C directly to 24°C (the rate of cooling was about 15°C/h). This experiment revealed that it was possible to produce films in glass surfaces, but most importantly, that the stepwise CTR was not essential. The important was to guarantee a slow cooling process (slower than at the room temperature).

Figure 73 (A-F) shows the aspect of the films produced in containers having distinct bottom areas. Films were very thin, transparent, with some elasticity and resistant enough to be removed in one piece. Because the flasks were sufficiently big, DNA/dendrimer films were also prepared with the triple amount of DNA per cm^2 , maintaining the concentration of solutions constant but using volumes three times higher. In this case, the films were white, even more easily to remove from the bottom of the flasks and very maneuverable (Figure 73, H).

When lyophilized on the top of a glass surface, the films become brittle and impossible to be removed from the substrate. However, those lyophilized in microcentrifuge tubes presented a higher elasticity and were shown to be more easily detached from the bottom, adopting its shape, with some curls on the edges (these films were prepared in 2 mL SC tubes, which have vertical walls and more planar bottoms; this allowed to have a lower bottom surface area and obtain thicker and more resistant films). These films can also be observed in Figure 73 (I-J).

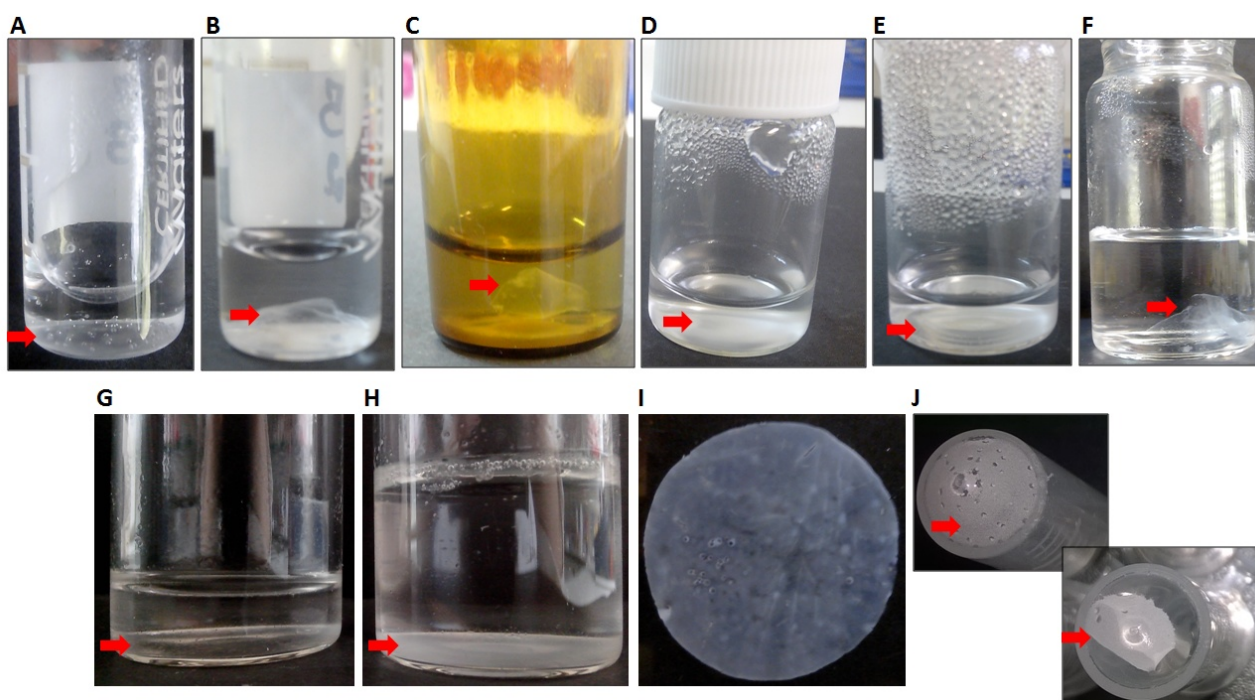


Figure 73 - Films based on SS-DNA and G5 PAMAM dendrimers (at a N/P ratio of 5), prepared in glass flasks using $472 \mu\text{g DNA per cm}^2$ of bottom surface, after a slow cooling process. Flasks had different bottom surface areas: (A) 1.06 cm^2 , (B) 1.06 cm^2 (after detachment), (C) 2.00 cm^2 , (D) 3.76 cm^2 , (E) 5.90 cm^2 , (F) 5.90 cm^2 (after detachment), (G) 5.90 cm^2 (film having $472 \mu\text{g DNA per cm}^2$, and (H) 5.90 cm^2 (film having $1416 \mu\text{g DNA per cm}^2$). Lyophilized films: (I) film having $1416 \mu\text{g DNA per cm}^2$, after detachment and lyophilization, and (J) films on the bottom of microcentrifuge tubes (top - attached to walls, bottom - detached and curled film). The red arrows are pointing the films on the bottom of the flasks.

3.3. Film formation using other polymers

After successfully preparing DNA/dendrimer-based films, we raised the hypothesis that they could possibly be prepared using other cationic polyelectrolytes, that is, polymers containing a high number of positive groups in their structure. Such attempt could, for example, result in a DNA film prepared with natural or other synthetic polymers, with better properties and more cost effective for future applications. Also, we wanted to know whether the architecture of the polymer (linear or branched) and their size (molecular weight) were crucial parameters for film formation. As such, attempts were made to prepare films using poly(ethyleneimine) – branched (brPEI-0.8kDa and brPEI-25kDa) and linear (10 kDa) - , poly(allylamine hydrochloride) (PAH, linear, 56kDa), chitosan oligosaccharide (COL, linear, 5kDa) and protamine from salmon (4.2 kDa). Curiously, using the developed and optimized protocol for DNA/dendrimer-based film preparation, from all polymers tested, films were only obtained when high molecular weight branched PEI (br PEI-25kDa) was used.

When low molecular weight brPEI-0.8kDa solution was added to SS-DNA solutions after heating, cooling down using a CTR resulted in clear solutions and no film formation. In fact, the molecular weight of brPEI-0.8kDa is lower than that of G1 PAMAM dendrimer (1.4 kDa) for which films were not formed too. On the contrary, brPEI-25kDa has a size comparable with G5 PAMAM dendrimer (28.8 kDa) and approximately 200 primary amine surface groups. In this case, film formation was consistent with the similarity between these two molecules. When linear PEI was used, the solution become turbid after the CTR cooling process and a thin layer of white particles was visible in the bottom of the microcentrifuge tube. However, films were not produced in this case, even with a molecular weight in between the molecular weight values of G3 and G4 PAMAM dendrimers for which films could be produced. This finding clearly shows that the architecture of the polymer is important for film formation. The spherical (or near-spherical) shape of the molecules seems to be an important point for the process to be successful. Indeed, for a given volume, the object with the smallest surface area is the sphere. So, for equivalent molecular weights, charge will be more concentrated in spherical molecules. Indeed, the previous experiments using dendrimers already showed the importance of electrostatic interactions in the process but, from these results, one can further conclude that charge density is an important factor in film formation. Aswell, the different molecular architecture would result in different arrangements with DNA. The experiments with the other cationic polyelectrolytes, namely PAH and COL also failed, being these results consistent with the ones obtained using PEI. Using chitosan, immediate precipitation was observed, with a cloth floating in solution.

Protamine is a naturally occurring peptide existing in the sperm (Figure 74). It is an arginine-rich protein known for being deeply involved with DNA condensation in densely and highly packed structures⁵⁸⁵.

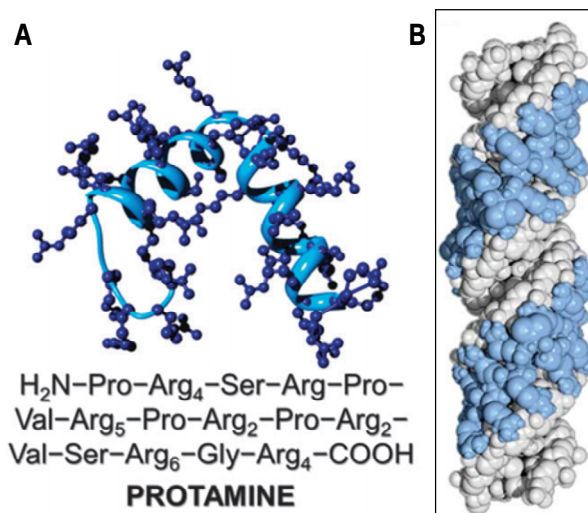


Figure 74 - Protamine structure and DNA binding positioning. (A) Typical protamine aminoacid sequence and structure⁵⁸⁶. (B) Protamine (in blue) binding to DNA's (grey) major groove, wrapping around the DNA helix, neutralizing the phosphate backbone⁵⁸⁷.

The binding of protonated arginine residues to DNA's phosphate groups is base-sequence independent, contrary to the histones⁵⁸⁸. Due to the high content in arginine residues ($\text{pK}_a = 12.5$), protamine is a very basic peptide that contains many positively charged guanidinium groups that are protonated at acid and basic pH values ($\text{pH} < 12.5$). When protamine was used to prepare films with SS-DNA, a gel-like transparent layer was formed in the bottom of the microcentrifuge tube, almost forming a film. However, this gel-like layer was very fragile and could not be removed from the container. Indeed, the size of protamine is between those of G2 and G3 dendrimers, which may explain this result. Another factor could be temperature-induced denaturation, which may alter its structure, thus, the way it interacts with DNA.

3.4. Characterization of the DNA/dendrimer-based films

3.4.1. Presence of SS-DNA and dendrimers inside films

In order to visually confirm the presence of SS-DNA in the films, a SS-DNA film was prepared with G4 PAMAM dendrimers ($\text{N/P} = 5$). Then, it was detached from the bottom of the glass vial with the help of a micropipette tip. Afterwards, in the same vial, the film was placed in contact with ethidium bromide (EtBr) solution (pink-orange color). As mentioned before, EtBr is an organic molecule that can intercalate between the base pairs of DNA, emitting a strong fluorescent signal.

Upon being in contact with EtrBr solution, the film gradually became pink-orange stained while the solution gradually lost its color. Figure 75 shows some images of this film after EtBr staining exposed to normal light and UV/blue radiation. These results undoubtedly show that the film contains DNA.

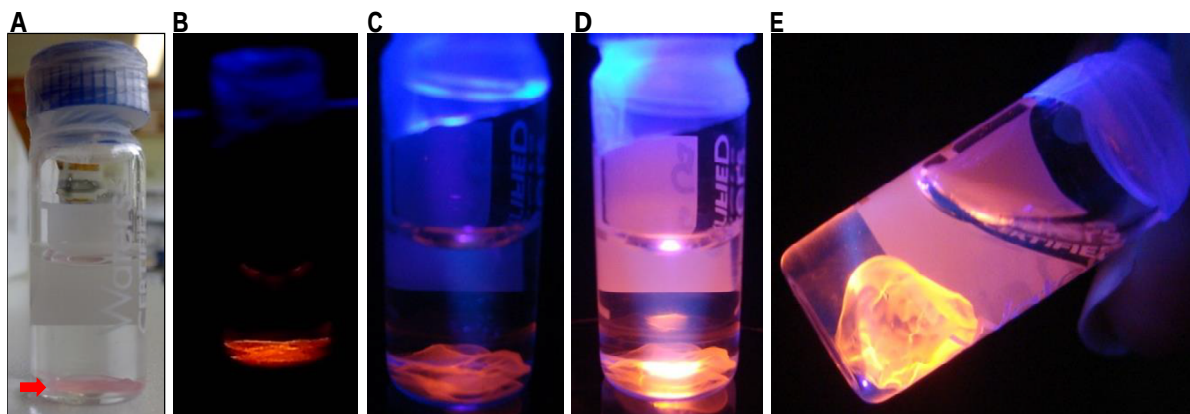


Figure 75 - Presence of SS-DNA in the film prepared with G4 PAMAM dendrimer (at a N/P ratio of 5). (A) EtBr stained DNA film (pale pink-orange) in the bottom of the glass vial (indicated by the red arrow). (B) Stained DNA film inside a UV inspection cabinet (dark orange), (C) near the fluorescence microscope blue light beam (330 - 380 nm), (D) on the top of the blue light beam, (E) after moving the film by shaking the flask (film is bright orange-yellow).

The presence of the PAMAM dendrimers inside the films was also confirmed. For that goal, a SS-DNA film was prepared with G4 PAMAM dendrimers labelled with rhodamine isothiocyanate. First, dendrimers were conjugated to RITC using an adequate synthesis process schematized in (Figure 76). The success of this reaction was verified by UV-Vis spectroscopy. In fact, the analysis of the absorption spectra of RITC solutions (Figure 76, C) shows a maximum at 556 nm. After being conjugated to the dendrimer, the maximum of absorption of RITC shifted to higher wavelengths (560 nm), a phenomenon known as red or bathochromic shift, which indicates that the conjugation was successful. The quantification of the number of RITC molecules per G4 PAMAM dendrimer was determined measuring absorbance at 556 nm and using a RITC standard calibration curve (Figure 76, D). After this characterization, the G4-RITC dendrimer was used to prepare DNA/dendrimer films that, thus, presented a pink color and orange-red fluorescence (Figure 76, B).

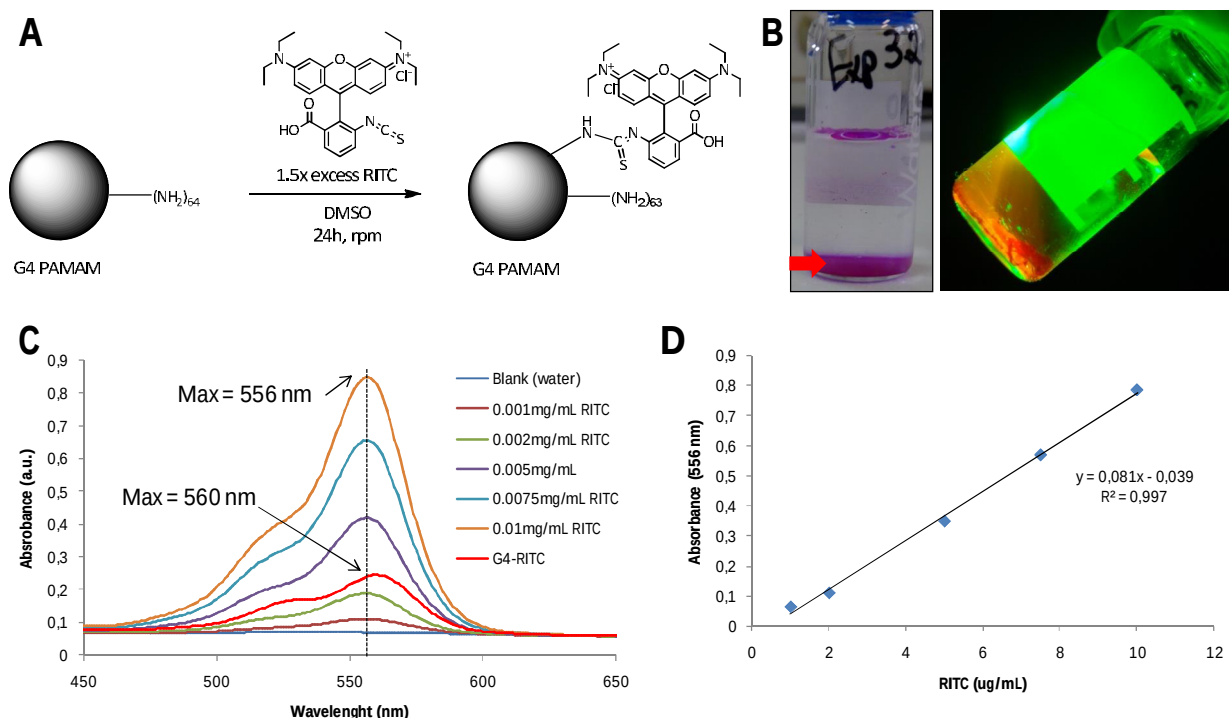


Figure 76 - Preparation of fluorescent labeled PAMAM dendrimer, characterization and film preparation. (A) Reaction scheme of the preparation of RITC-labeled G4 PAMAM dendrimers. (B) Pink-fushia stained DNA film on the bottom of the vial (indicated with red arrow), and on top of the green light beam (510 - 540 nm) of afluorescence microscope (film is bright orange-red). (C) Absorbance spectra of blanck, RITC solutions at several known concentrations and RITC-modified G4 PAMAM dendrimer. (D) RITC calibration curve for quantification.

3.4.2. DNA/dendrimer-based film stability at extreme pH values

The stability of the DNA/dendrimer films after an extreme pH variation was studied. To attain this objective, films were prepared based on G5 PAMAM dendrimers at N/P ratios of 3, 4 and 5. Then, a small volume of acid (5M HCl) and base (6M NaOH) solution was added to the films. The results of these experiments were in agreement with the conclusions achieved in the previous assays. In all cases, films did not dissolve in the presence of an acidic solution which was expected since dendrimers keep their protonated state in an acidic environment. On the contrary, when an alkaline solution was added, the films dissolved very quickly (instantaneously) and completely disappeared (the solutions became clear/transparent, without any particles in suspension). This can be explained by dendrimer deprotonation under alkaline conditions (pH values well above 10). In addition, DNA may suffer denaturation at extreme alkaline pH values due to the interruption of hydrogen bonds between the nucleobases^{12,15,17}. As the DNA/dendrimer film driving forces were disrupted, the film dissolved. The process was captured at the microscope, where films were placed in glass coverslips and were then separately subjected to the addition of concentrated alkaline and acidic solutions (Figure 77).



Figure 77 - Behaviour of a DNA/dendrimer film based on G5 PAMAM dendrimer (N/P ratio 4) under extreme pH changes. (A) Film without any solution, (B) after concentrated HCl addition (the film does not dissolve), (C) after concentrated NaOH addition (red arrow indicates immediate dissolution).

3.4.3. Scanning electron microscopy (SEM) of DNA/dendrimer and DNA/PEI-based films

The morphology of the DNA/dendrimer-based films was analyzed in more detail using scanning electron microscopy (SEM). The films were first observed in a benchtop SEM using a charge reduction sample holder. Figure 78, Figure 79, and Figure 80 show representative images of the morphology of DNA films prepared with G3, G4 and G5 PAMAM dendrimers (N/P ratio 4), respectively. Figure 81 shows images acquired for the films prepared with brPEI-25 (branched PEI with 25kDa, N/P ratio 4). Both top and bottom film surfaces of lyophilized films were visualized.

Curiously, in all DNA films, the top surface (side in contact with the solution) has a distinct morphology from the bottom surface (side contacting with the plastic or the glass). The top surface is composed of spherical particles (in the micron range), homogeneously distributed across the surface. These particles are connected to each other, reminding sintered particles, like those that may form in metals, ceramics or plastics⁵⁸⁹ (since they are fused with each other at contact points), or even polymeric spherulite structures^{590,591}. The particles that are seen in the top surface seem to emerge from a flat, compact and homogenous material in the bottom. In some of the images, it is possible to see both sides of the films since cracks (cross-section) images in the lyophilized films were observed. The morphology was always the same, independently of the polymer, or of the generation of dendrimer used. SEM images obtained in a FEG-SEM equipment allowed the observation of these surfaces with higher resolution and magnification. In this case, only the top surface was visualized. Examples are shown for DNA films prepared with G3 PAMAM dendrimers and brPEI-25 (N/P ratio 4) (Figure 82 and Figure 83, respectively). The conclusions that can be taken from these images are the same, that is, the surface is made of interconnected particles.

So, in general, SEM images might reveal us the possible mechanism of film formation, which may involve two consecutive steps. It looks like a first layer of material is deposited in the bottom of the container and that a second layer composed of interconnected particles is after deposited in the top.

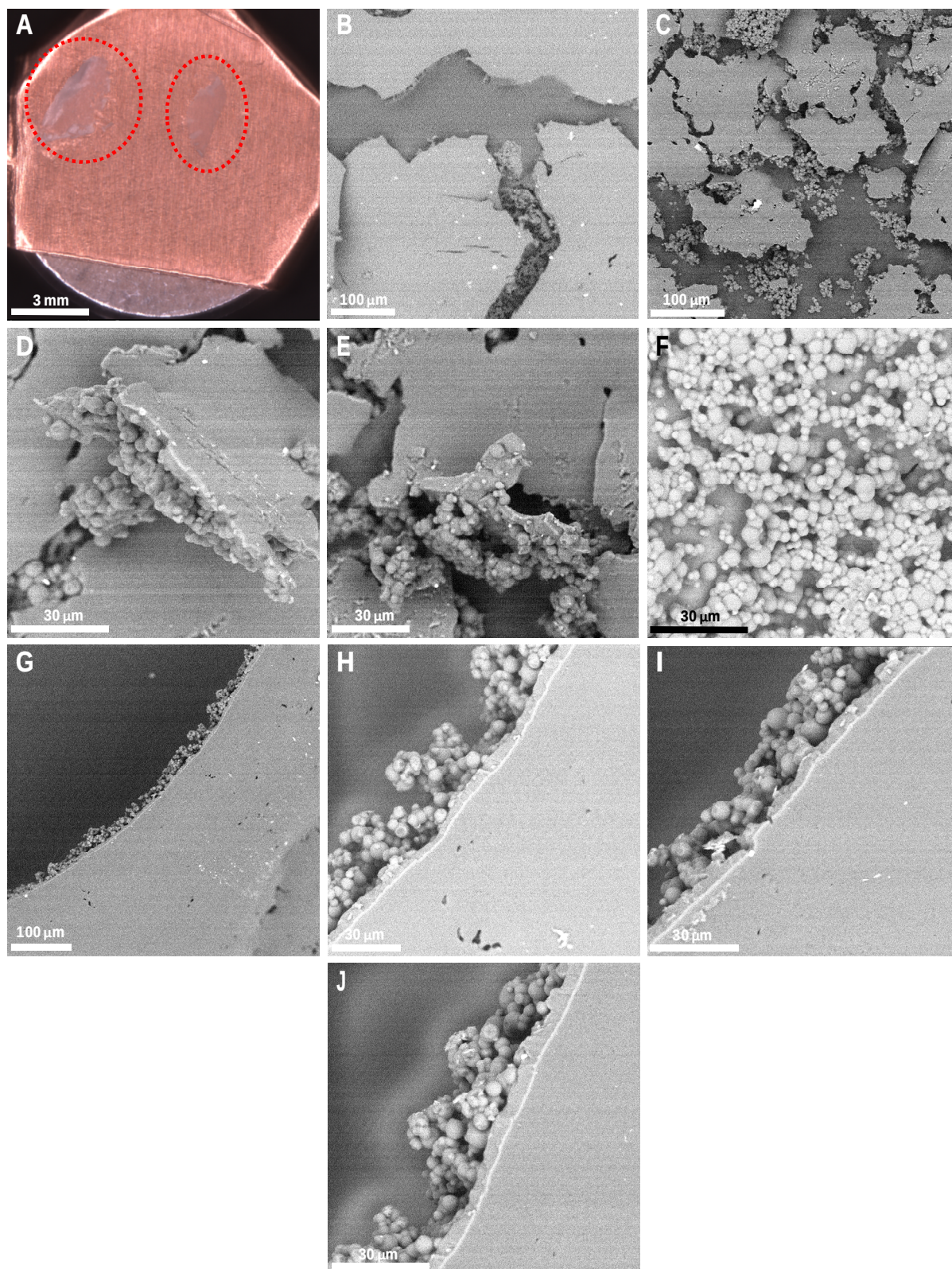


Figure 78 - SEM images of a lyophilized DNA film based on G3 PAMAM dendrimers (at a N/P ratio of 4). (A) Film on the top of the copper tape (20x), (B) Flat compacted side with cracks exposing particles from the top of the film (550x), (C) Fragmented film (640x), (D) Cross-section of the film (2800x), (E) Cross-section of film (2250x), (F) Spherical particles in the top of the film (2800x), (G, H, I and J) Cross-section of the film at different magnifications (500x, 2000x, 2500x and 2800x, respectively).

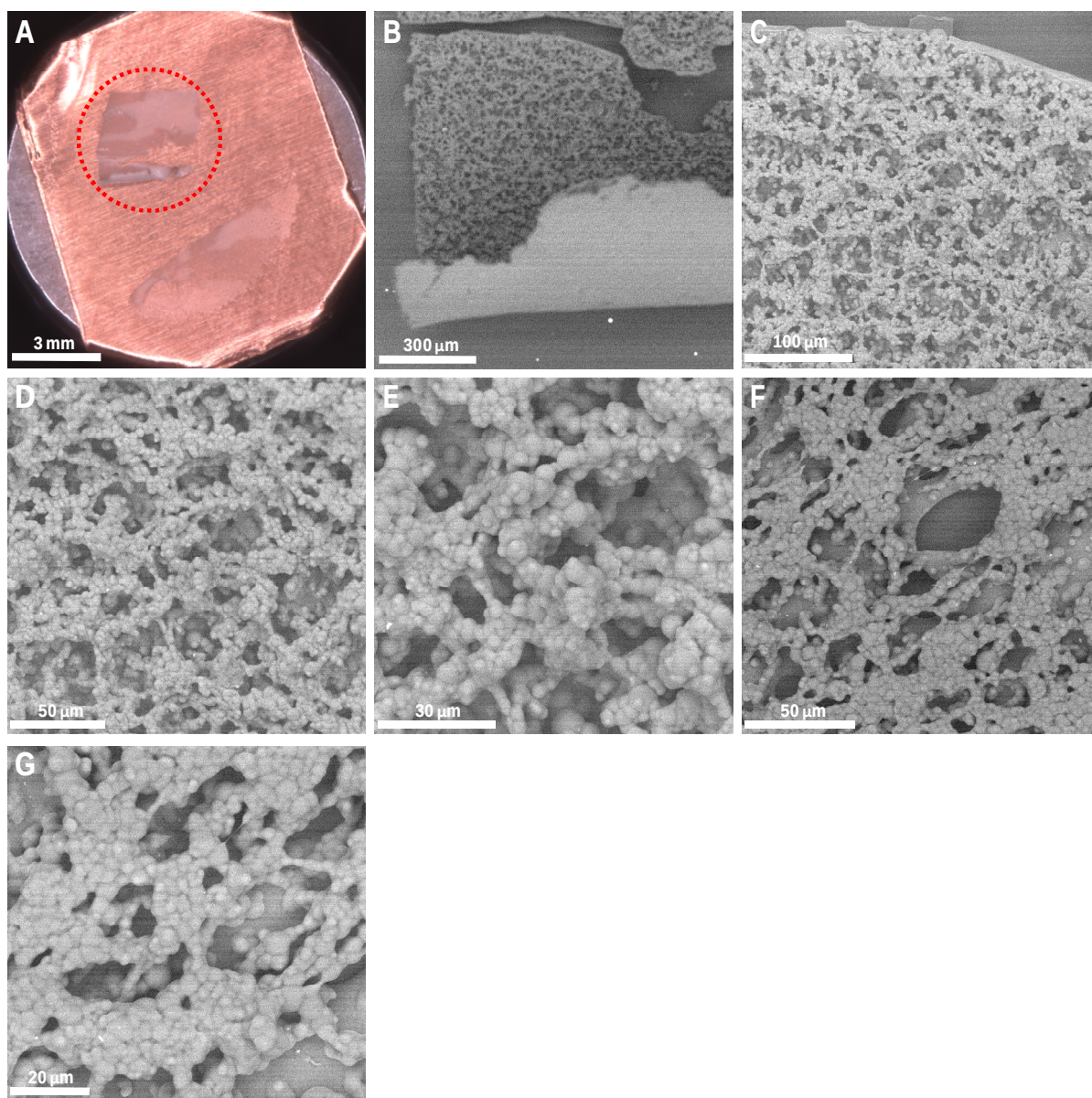


Figure 79 - SEM images of a lyophilized DNA film based on G4 PAMAM dendrimers (at a N/P ratio of 4). (A) Film on the top of the copper tape (20x), (B) Curled film, exposing both sides (the flat compact side and the particulated side) (240x), (C) Edge of the DNA film (780x), (D, E, F and G) Particles in the top of the film (1400x, 2900x, 1650x and 2950x respectively).

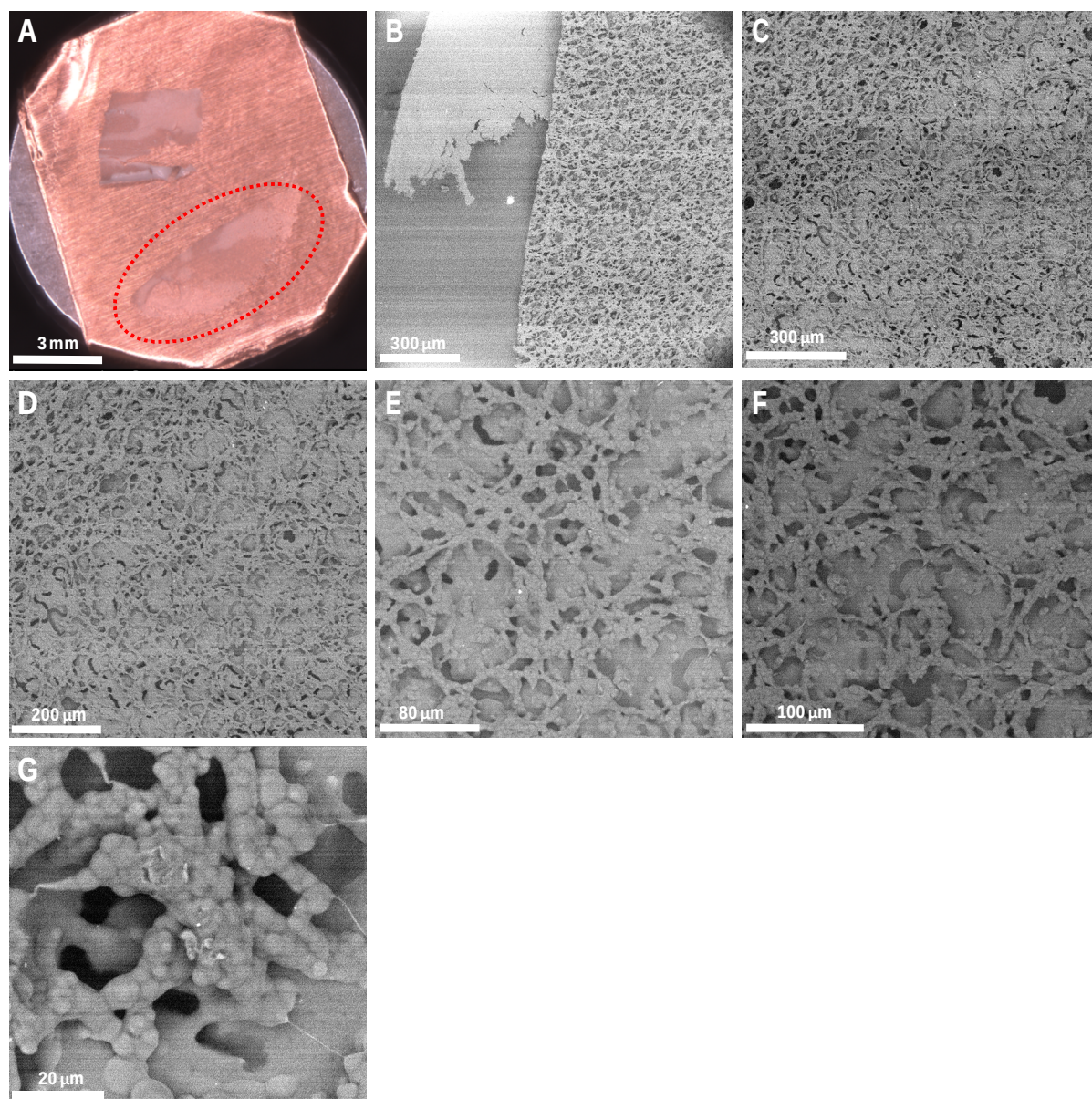


Figure 80 - SEM images of a lyophilized DNA film based on G5 PAMAM dendrimers (at a N/P ratio of 4). (A) Film on the top of the copper tape (20x), (B) Curled film, exposing both sides (the flat compact side and the particulated side) (195x), (C, D, E, F and G) Particles in the top of the film (245x, 330x, 930x, 870x and 3400x, respectively).

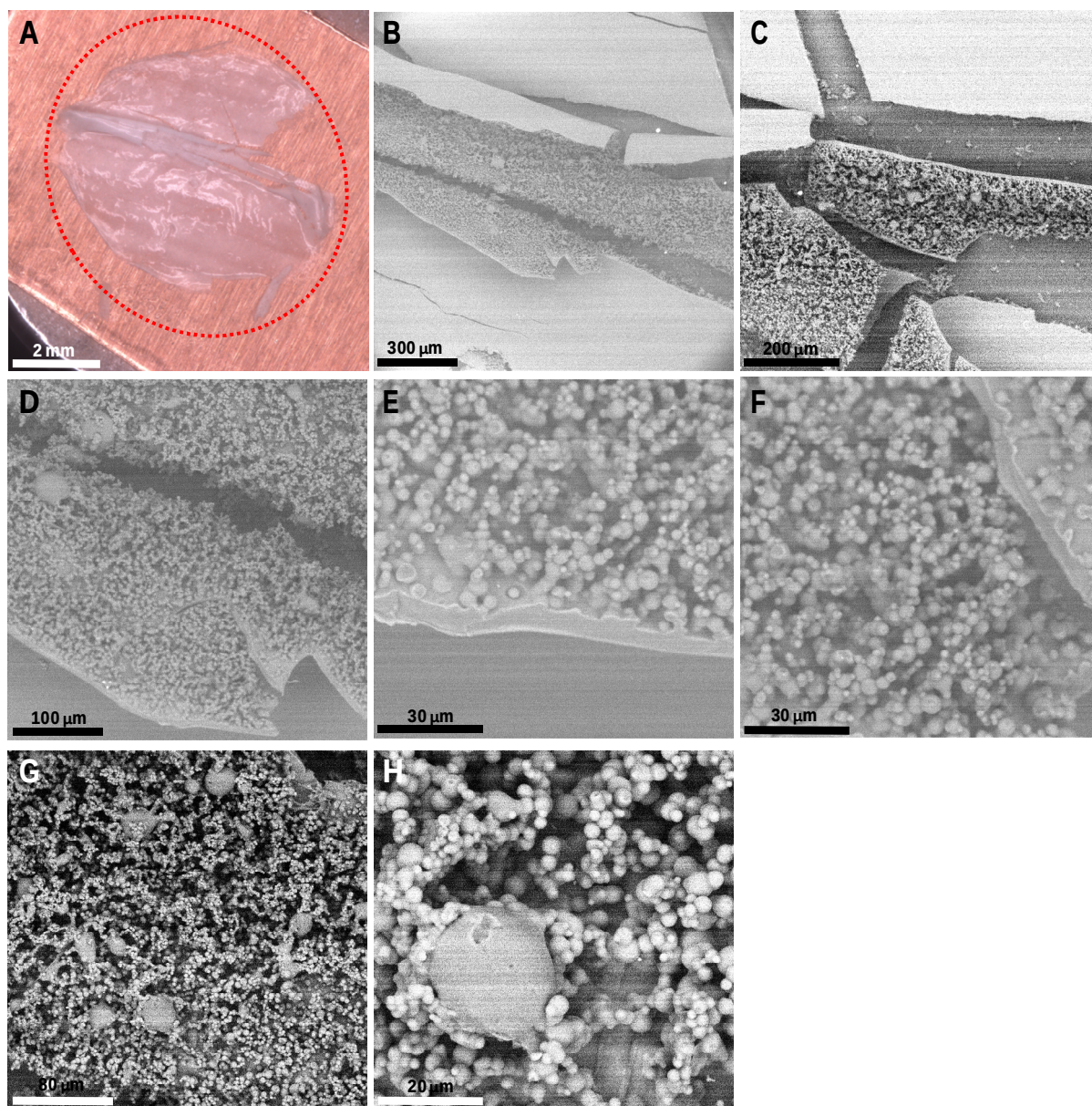


Figure 81 - SEM images of a lyophilized DNA film based on brPEI-25 (at a N/P ratio of 4). (A) Film on the top of the copper tape (31x), (B, C and D) Cracks exposing particles (195x, 350x and 670x), (E, F) Cross-section of the film exposing both sides, with visible particle fusion (2600x), (G, H) Particles with different size are present in the top surface (950x and 3500x, respectively).

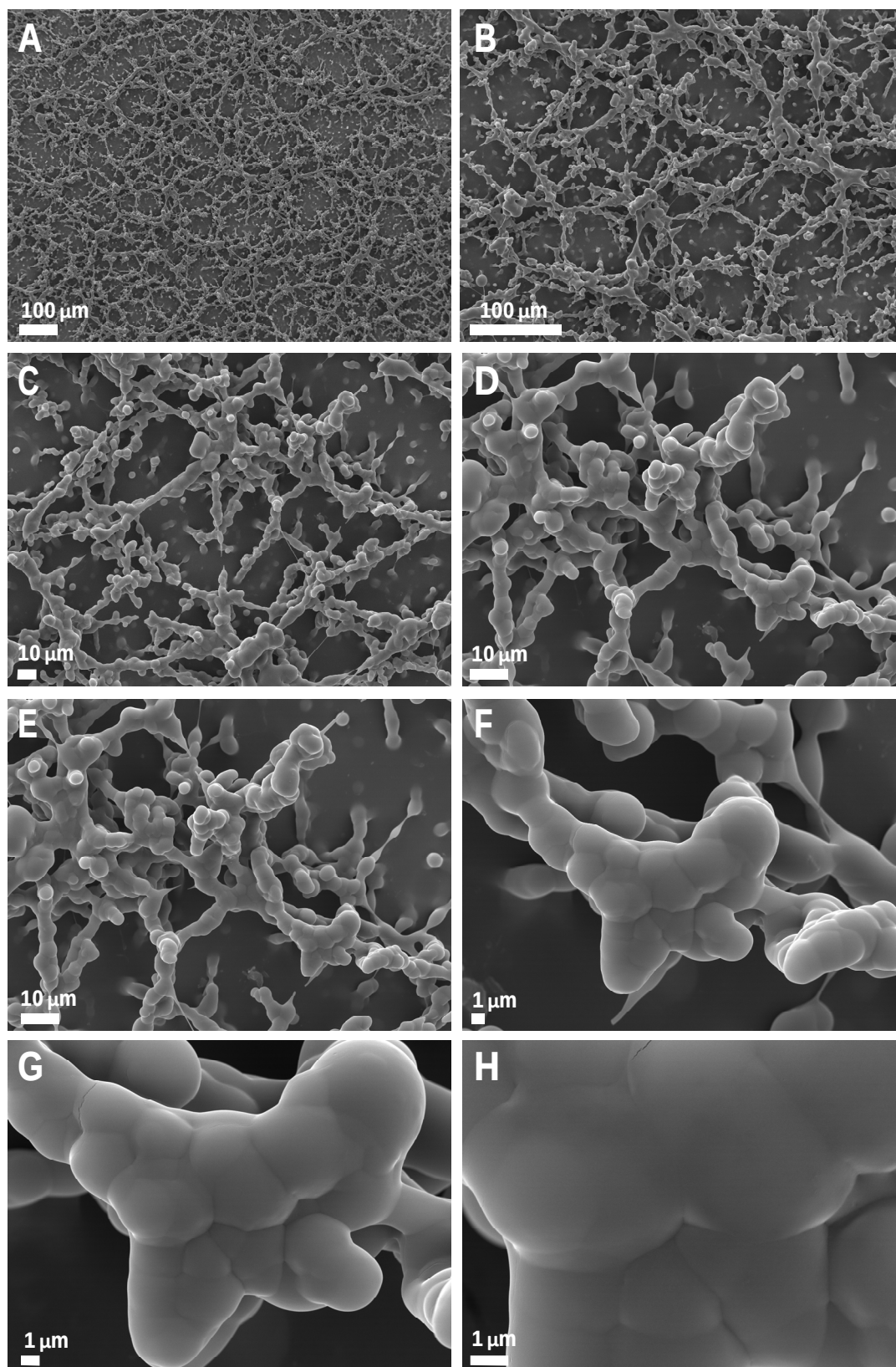


Figure 82 - Scanning electron microscopy images of a lyophilized DNA film based on G3 PAMAM dendrimers (at a N/P ratio of 4) with increasing magnifications. (A) 100x, (B) 250x, (C) 500x, (D) 1000x, (E) 3000x, (F) 5000x and (G) 10000x.

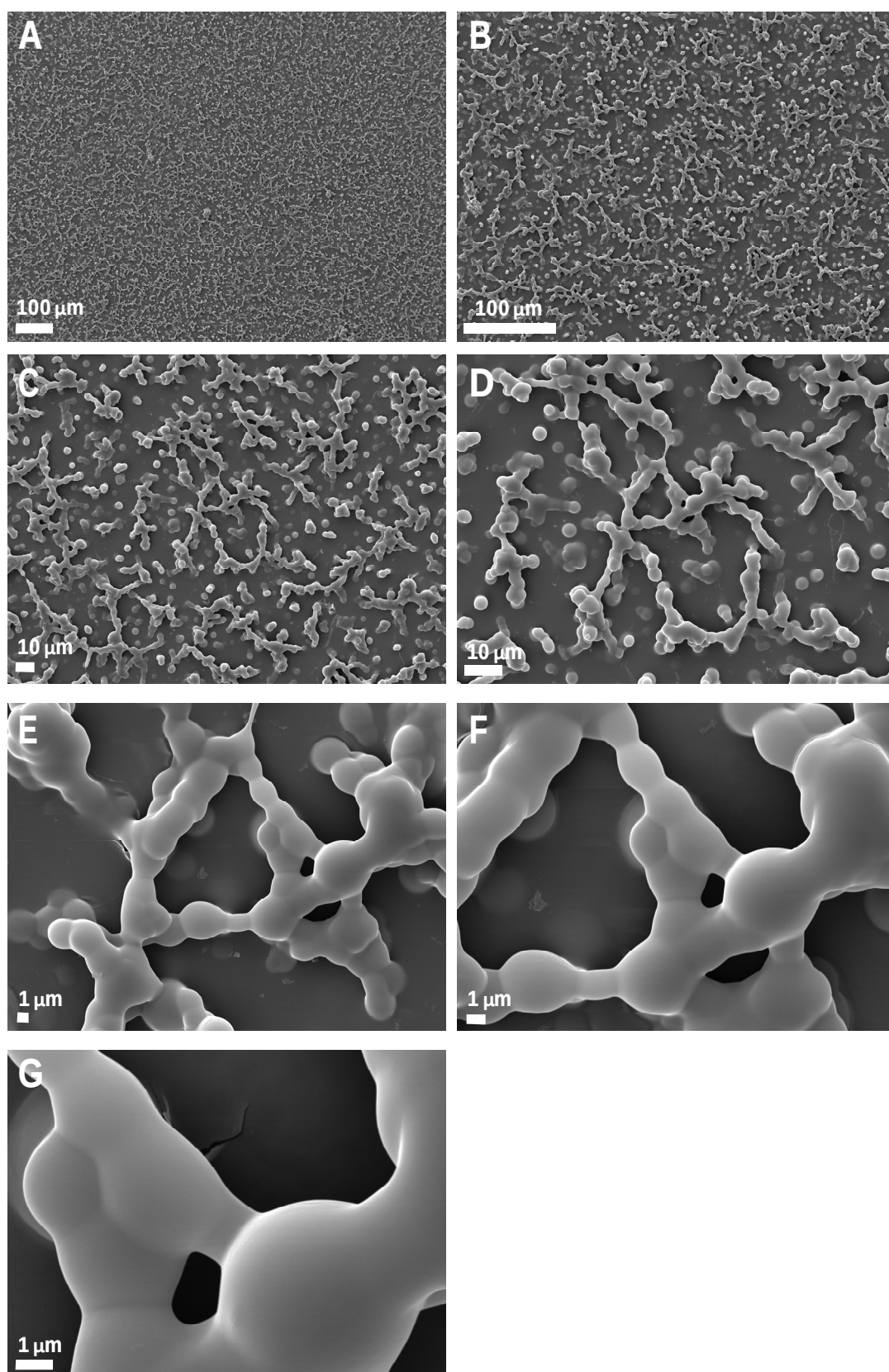


Figure 83- Scanning electron microscopy images of a lyophilized DNA film based on brPEI-25 (at a N/P ratio of 4) with increasing magnifications. (A) 100x, (B) 250x, (C) 500x, (D) 1000x, (E) 3000x, (F) 5000x and (G) 10000x.

Since it is known from the literature that DNA suffers crosslinking under UV irradiation⁴⁵⁰, the effect of exposing DNA/dendrimer films to UV light for 6 h, 24 h and 48 h was also evaluated by SEM. Determine whether the UV-radiation changes the film surface is important, for potential biomedical application, since materials like these can be sterilized by UV radiation. For this purpose, DNA films based on G3 PAMAM dendrimers were used at N/P ratio of 4. Interestingly, after being prepared, the films completely covered the glass coverslip but, upon UV irradiation, they shrunk. As new covalent bonds were formed, the distance between DNA strands decreased leading to film shrinkage. Figure 84 shows the morphology of these films that look different from the non-irradiated samples. An increase in the irradiation time results in surface damage which is more visible at low magnification. It appears that UV irradiation affects particles at the film surface as new bonds are created. Clearly, the surfaces gradually lose their homogeneous aspect.

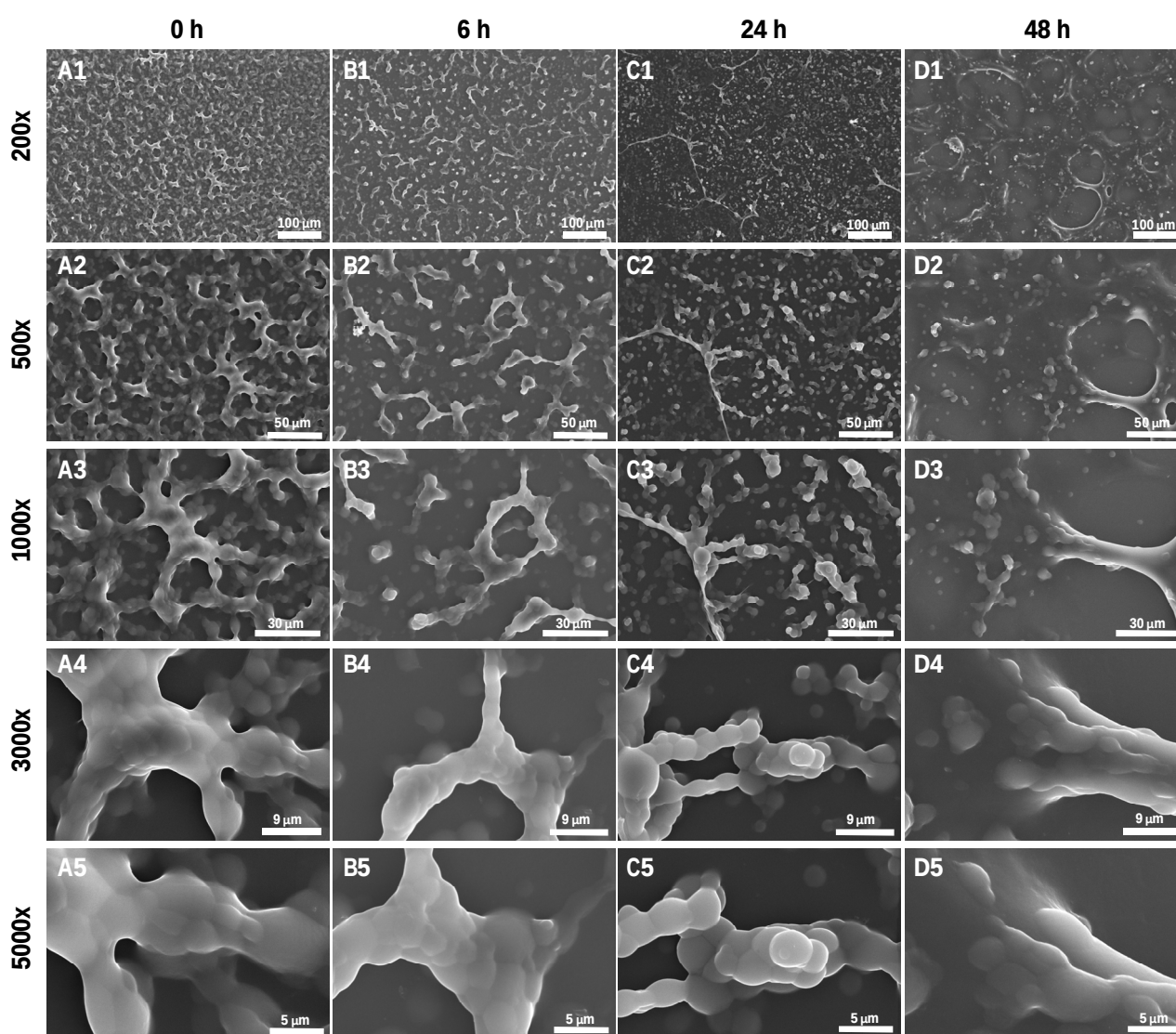


Figure 84 - Scanning electron microscopy images of a lyophilized DNA film based on G3 PAMAM dendrimers (at a N/P ratio of 4). Letters indicate different UV-irradiation time, namely (A) 0h, (B) 6 h, (C) 24 h and (D) 48 h. Numbers indicate increasing magnifications (200x, 500x, 1000x, 3000x, 5000x).

3.4.4. Films' surface zeta-potential (ZP)

The surface zeta-potential (charge) of DNA films prepared with G3, G4 and G5 PAMAM dendrimers was measured. Indeed, the zeta-potential may provide useful information to understand the nature of the produced DNA films. Also, since the idea is to use the films for biomedical purposes, the evaluation of the surface zeta-potential is important as it may have a huge impact on cell adhesion and viability. For example, it is reported that dendrimers' positive charge is responsible for their toxicity due to interactions with the negatively charged cell membranes⁵²⁷.

For surface zeta-potential analysis, thicker films were prepared for easier manipulation (the triple amount of DNA was used in relation to the usual procedure). The positioning and immobilization of the film in the sample holder was possible using parafin wax at the lowest temperature possible, so that the temperature would not affect the DNA in the film. After positioning the clamping cell, and as the electrolyte solution (KCl) passed through the channel between the DNA film and the clamping cells, the DNA film surface charge or ZP was measured. Results are shown in Table 3.

The surface charge was positive for all DNA films. This was expected once there is an evident excess of positive charge in the films (the N/P ratio is above 1). Films prepared at the same N/P ratio showed similar values for zeta-potential, independently of dendrimer's generation. However, due to the high errors associated with the measurements, the results do not allow for the establishment of a relation between ZP and the increase in N/P ratio for films prepared with dendrimers of the same generation.

Table 3 - Zeta-potential for DNA films prepared with PAMAM dendrimers of different generations, at several N/P ratios. The pH of the solution used for the measurement is indicated. Shaded lines indicate films at the same N/P ratio 4. Results are represented as mean $\pm$ s.d., with n = 3.

	pH	ZP (mV)
DNA/G3 (N/P=4)	5.15 $\pm$ 0.03	24 $\pm$ 5
DNA/G4 (N/P=4)	5.32 $\pm$ 0.01	22 $\pm$ 5
DNA/G5 (N/P=3)	5.59 $\pm$ 0.02	28.3 $\pm$ 0.5
DNA/G5 (N/P=4)	5.66 $\pm$ 0.06	22 $\pm$ 12
DNA/G5 (N/P=5)	5.63 $\pm$ 0.01	21 $\pm$ 7

3.4.5. Circular dichroism (CD) measurements

Circular dichroism measurements were performed to analyze the changes in SS-DNA's conformation after the process used to prepare the DNA/dendrimer films. During this procedure, DNA is heated above its T_m , which should result in denaturation. That is, the native DNA B-form conformation (B-form is the most common DNA's conformation) will be changed. CD is a technique based on absorbance. It measures the difference in absorbance of left and right circularly polarized light, $\Delta A = A_L - A_R$, in samples containing one or more chiral groups⁵⁹². Because of that, if a molecule absorbs more left than right circularly polarized light, the CD signal is positive, otherwise, the CD signal will be negative. In biomacromolecules, such as proteins and DNA, along with the individual residue/building block absorbance, the signal is mainly influenced by the tridimensional structure of the molecule^{592,593}. CD spectroscopy is often applied in protein studies, such as in secondary and tertiary structure estimation, analysis of conformational changes in specific conditions, and evaluation of denaturation, ligand interactions and kinetics parameters. Furthermore, this technique has been also used to understand DNA structure and interactions⁵⁹⁴. There are several published works where the CD technique is used to analyze the effect of temperature, hydration, pH, ions, ligand/complexes on DNA conformation, for both ss or dsDNA⁵⁹⁵⁻⁵⁹⁹.

The CD signal for DNA is attributed to the symmetric/perpendicular bases arrangement/stacking relatively to the helix axis and to the asymmetric carbons in the backbone sugars⁶⁰⁰. Transitions in the sugars are mainly $\sigma \rightarrow \sigma^*$ (at around 190 nm), with weak contribution for the CD signal⁶⁰¹. The proximity of the stacked bases in DNA allow for the $\pi \rightarrow \pi^*$ (higher intensity) and $n \rightarrow \pi^*$ transitions (lower intensity), originating a CD signal (below 300 nm). Because of that, the CD signal is different for every DNA conformation, namely, A, B and Z-forms, as well as G-quadruplexes, C-motifs and triplexes^{594,600,602}. For example, the typical A-, B- and Z-forms of DNA have distinctive CD signal patterns⁵⁹⁹. The A-form of DNA (right-handed helix, shorter and compacted structure) presents a dominant positive band at 260 nm and a negative band at 210 nm, whereas its B-form (right-handed helix, medium compacted structure) has a 260 - 280 nm broad positive band and a negative band around 245 nm^{598,602}. The least common DNA Z-form (left-handed helix, looser structure) presents a negative band at 290 nm, a positive band at 260 nm and a deep negative band at 205 nm.

Circular dichroism is a very sensitive technique that can be used at low and high DNA concentrations, as well as with short and long DNA molecules. It is reported that it can also be used with films⁶⁰². As such, in the present work, CD measurements were performed using DNA/dendrimer films based on G5 PAMAM dendrimers at N/P ratios of 3, 4 and 5. For the analysis, the films (almost

completely transparent, prepared with $472 \mu\text{g}/\text{cm}^2$) were placed in an appropriate cuvette, as flat as possible. Several controls were also considered. Results are summarized in Figure 85.

The first measurements were done using a 1 mg/mL SS-DNA solution (this is the concentration used for film preparation) prepared in PBS which was analyzed as such (native DNA) and after passing through the heating/cooling program used for film preparation (Figure 85, A). The objective, here, was to evaluate the influence of the heating/cooling process on DNA's conformation. The spectrum acquired for native DNA showed that the SS-DNA adopts a B-form conformation in solution, with a positive band at $\approx 270 \text{ nm}$ and a negative band around 240 nm . After the heating/cooling process, similar bands were observed which revealed that the hybridization process occurred without significant changes in DNA's conformation.

When SS-DNA films based on G3 and G4 PAMAM dendrimers were analysed by the CD technique, results were surprising (Figure 85, B2-C2). The bands corresponding to SS-DNA B-form conformation were not detectable and a very intense and broad positive peak appeared above 290 nm , with a maximum at $\approx 300 \text{ nm}$ followed by a tail or shoulder. The intensity of the bands seemed to be N/P ratio-dependent, although the results obtained did not allowed the definition of a clear trend between CD signals and the N/P ratio values. According to the literature⁶⁰², the signals above 300 nm does not come from the DNA secondary structure, because DNA does not absorb above 300 nm . Instead, this signal is attributed to light scattered by particles formed by condensed DNA. These particles have been compared as chromosome analogues since they possess a regular tertiary structure. It is reported that, when regularly packed, condensed DNA gives a signal in CD which is characterized by either positive or negative bands with extremely large amplitudes. In fact, several works reveal that the CD technique can distinguish ordered condensation from random aggregation⁴⁷. These particles with regularly packed and organized DNA have been described in literature as psi-form (ψ -form) or DNA wires. As a conclusion, the formed DNA/dendrimer films should have organized DNA structures in their composition that are responsible for the CD signals observed. The positive signal shows that the particles are optically active towards right circularly polarized light, and this has been mentioned to be directly related chiral condensates possessing large-scale right oriented helicity. This is a phenomenon known by supramolecular chirality⁶⁰³, where intermolecular non-covalent interactions are established, resulting in a self-assembly and autonomous organization of the components.

In the spectra obtained for SS-DNA films based on G5 PAMAM dendrimers (Figure 85, D2), the signal above 290 nm observed for a N/P ratio of 5 is different and with a smaller amplitude. For the other N/P ratios, the bands seen appear at lower wavelengths and do not have the shape of the bands observed for the films based on G3 and G4 PAMAM dendrimers. Probably, the SS-DNA condensation achieved with the G5 PAMAM dendrimers is not as much ordered as for G3 and G5

PAMAM dendrimers. It should be noticed that control experiments were done with solutions only containing dendrimers (results not shown) where no signals were detected since these molecules are achiral³⁴⁷. Also, the supernatants of the films were also analysed to discard the possibility of signals coming from species in solution (Figure 85, B1-D1). In fact, only a residual signal was observed for supernatants, revealing that the high amplitude bands present in the CD spectra of the films come from the films themselves.

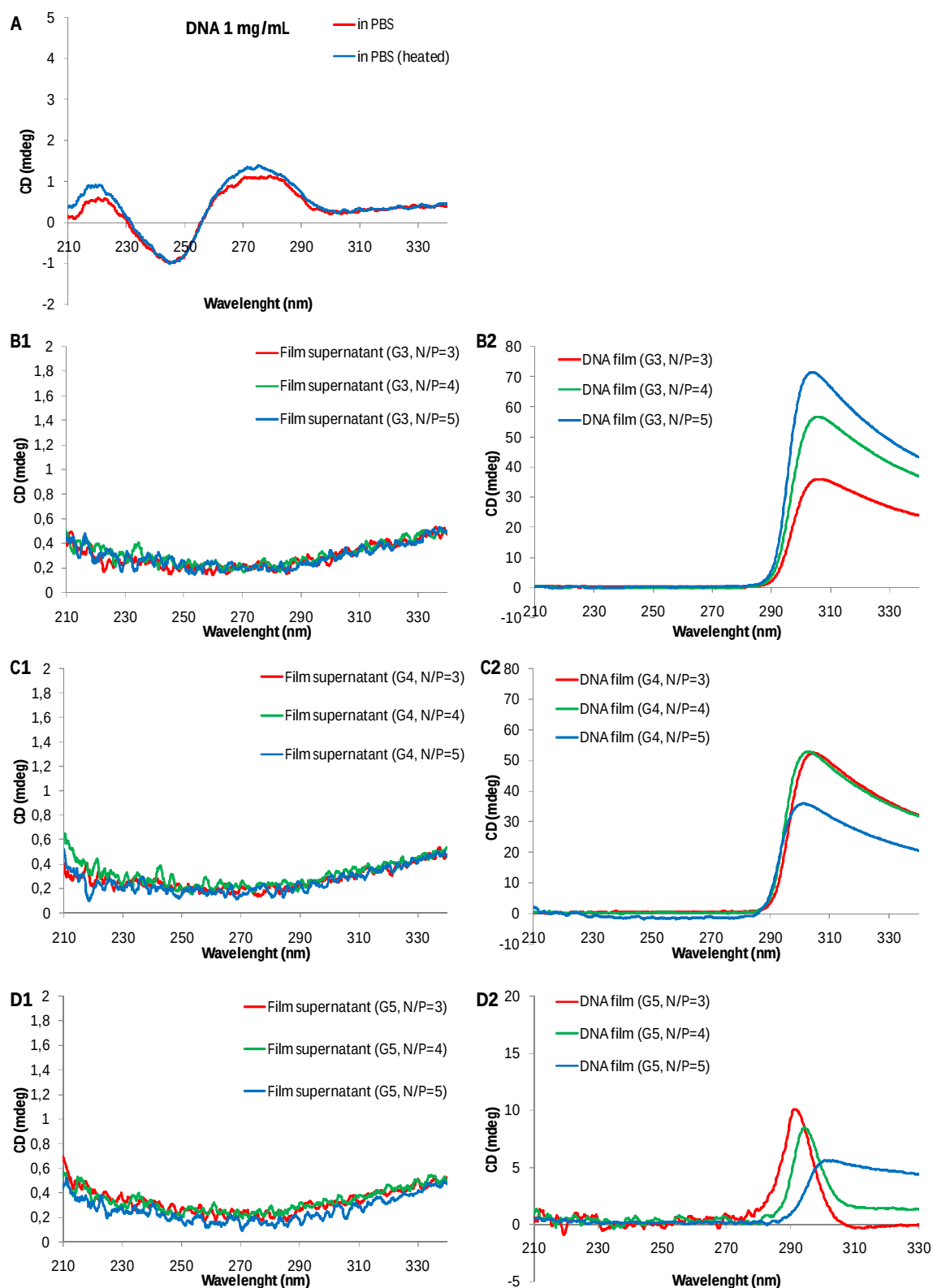


Figure 85 - Circular dichroism (CD) measurements with different DNA films. DNA at (A) 1 mg/mL before and after the heating/cooling process. CD spectra of (B1) supernatant and (B2) film of SS-DNA/G3. CD spectra of (C1) supernatant and (2) film of SS-DNA/G4. CD spectra of (D1) supernatant and (D2) film of SS-DNA/G5. Notice that the vertical axes do not have all the same scale.

3.5. Stability of the DNA/dendrimer-based films

To better understand the nature of the DNA/dendrimer-based films and to evaluate the possibility of their use in biomedical applications, films' stability was studied *in vitro* in conditions mimicking the fluids in the physiological environment. To perform this study, several films were prepared from SS-DNA and G3, G4 and G5 PAMAM dendrimers, as well as from SS-DNA and brPEI-25, all at a N/P ratio of 4. Then, the initial amount of DNA retained in the films was indirectly determined by quantifying the remaining DNA in the supernatant. As can be seen in Table 4, from the 500 µg of sodium SS-DNA used for film preparation, almost all was incorporated in the films. This is an important information which correlates with the absence of signals in CD measurements when the supernatants obtained after film formation were analysed. Table 4 indicates the DNA content in the films for posterior degradation studies in the corresponding solutions. The average percentage of DNA incorporation was 97.7 ± 0.6 , 97.8 ± 0.3 , 98.5 ± 0.4 , and 99.3 ± 0.1 for, respectively, the films based on DNA/G3, DNA/G4, DNA/G5 and DNA/brPEI-25. The differences in DNA content among the films are very slight.

Table 4 - DNA content of the films used in posterior stability evaluation assays. Results are shown as a % in relation to the 500 µg of DNA used for their preparation. Results are represented in mean $\pm$ s.d., with n = 30. Statistical analysis with * $p < 0.0001$.

	DNA content in The different films (%)	Statistical
DNA/G3	97.7 ± 0.6	G3 and G5* G3 and PEI*
DNA/G4	97.8 ± 0.3	G4 and G5* G4 and PEI*
DNA/G5	98.5 ± 0.4	G5 and PEI*
DNA/PEI	99.3 ± 0.1	

After being prepared, the films were separately exposed to different solutions, namely H₂O, PBS solution, DMEM, 1% (v/v) FBS, 10% (v/v) FBS and complete DMEM (DMEM comp., that is, DMEM containing 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution). The stability of the DNA/dendrimer-based films was then determined by quantifying the amount of DNA released into the solutions at different time points, by UV-visible absorbance at 260 nm. Results of a representative experiment are presented in Figure 86. After analysing all the results, one can conclude that the degradation of both DNA/dendrimer-based films and DNA/brPEI-25-based films is

negligible. Indeed, the first films produced in the scope of this work were kept sealed, in water, inside glass vials, for more than 2 years and remained intact. So, these films were shown to be extremely stable along time. This also indicates that DNA condensation by the PAMAM and PEI offers protection against degradation by nucleases present in the serum. Experiments done in the presence of FBS seemed to result in slightly higher film degradation which was expected due to the presence of nucleases in these media.

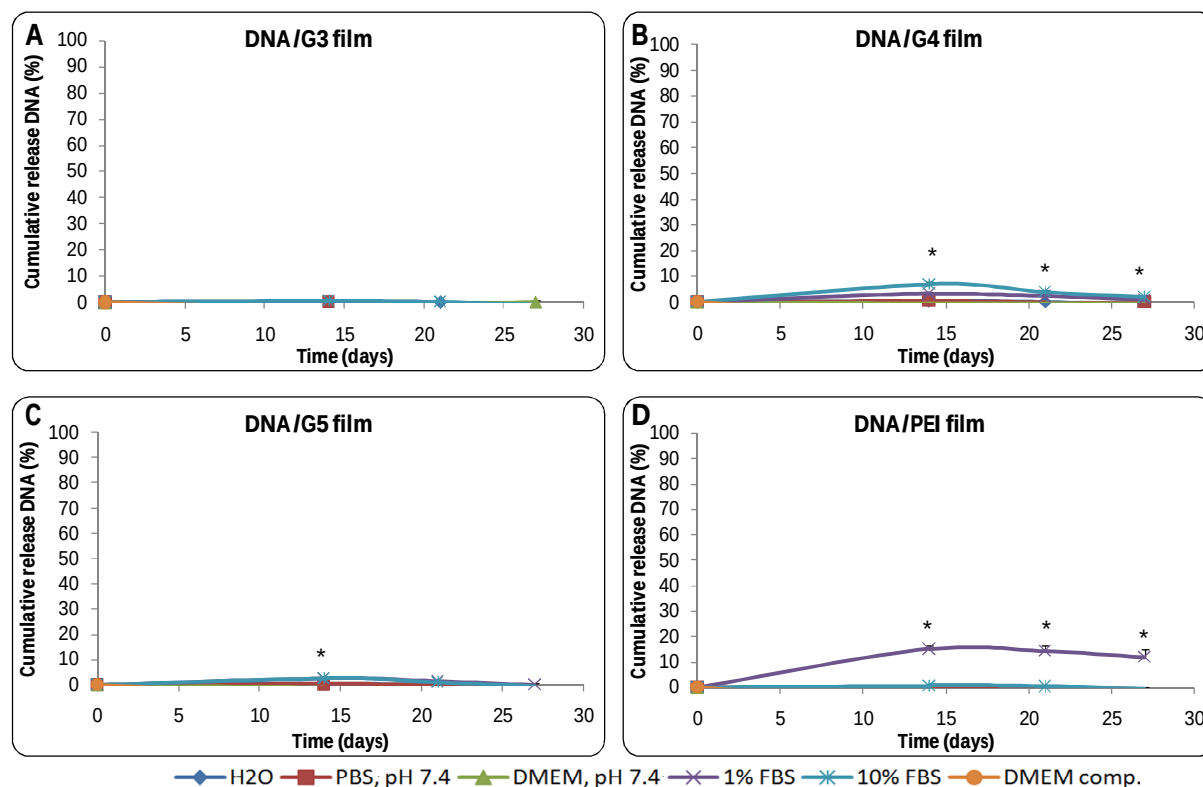


Figure 86 - SS-DNA release as a percentage of the amount of SS-DNA initially present in the film (representative experiment). DNA films were prepared with different polymers, at a N/P ratio of 4, and exposed to different solutions that mimic the fluids in the physiological environment, at 37°C. (A) DNA/G3 PAMAM dendrimer films, (B) DNA/G4 PAMAM dendrimer films, (C) DNA/G5 PAMAM dendrimer films and (D) DNA/brPEI-25 films. Results are represented as mean $\pm$ s.d., with $n = 5$. Statistical analysis compares serum-containing solutions (1% FBS and 10% FBS) with PBS pH = 7.4., with * $p < 0.0001$.

3.6. Proposal of a model for SS-DNA/dendrimer films' formation

The information gathered along the optimization process of film preparation and during films' characterization provided several clues that can help us to understand the nature of the forces behind these systems, or at least, propose a model for their formation. Indeed, from the previous results, one can conclude the following:

- a) Film formation depends on strong electrostatic interactions between SS-DNA and dendrimers. In fact, it was shown that the level of dendrimer protonation (stable films were only prepared when the initial pH of the solution was adjusted to 6) and the N/P ratio (stable films were only formed for N/P ratios in the range 3 to 5, independently of the dendrimer generation used) are crucial for film formation. Films were also shown to be very stable under extreme acidic conditions, for which the protonation of dendrimers is guaranteed. Under extreme alkaline conditions, the films dissolve due to deprotonation. Also, although the results obtained in the experiments done to evaluate the influence of magnesium ion on DNA condensation seemed to point out that this ion does not interfere with the electrostatic forces between DNA and dendrimers, because these studies were done at low concentrations, it is reasonable to think that the magnesium ion impairs film formation by binding to DNA's phosphate groups.
- b) Film formation depends on the extent of denaturation of SS-DNA. Indeed, it was observed that the maximum temperature initially applied to the SS-DNA solution was crucial for DNA film production which reveals the importance of the extent of DNA melting in the process.
- c) Film formation depends on partial hybridization of denaturated SS-DNA. Films are only produced when, after the melting of DNA, a slow cooling is applied, meaning that the system has more time for hybridization to occur. The agarose gel electrophoresis results also confirmed that hybridization took place, likely resulting in random crosslinking of the DNA molecules.
- d) While the films incorporate all SS-DNA used in their formation, due to the excess of charge (N/P ratio > 1), part of the dendrimers remain in solution. Possibly, dendrimers in the supernatant adsorb to the surface of films and, consequently, the zeta potential of the surface becomes positive.
- e) Films are formed of two distinct layers: one facing the supernatant which contains interconnected spheres, and another facing the bottom of the tube/flask which is composed of a more compacted material, as revealed by SEM analysis.
- f) Not only the charge, but also charge density is an important issue in film formation. Linear cationic polyelectrolytes, low generation dendrimers (G1 and G2), low molecular weight PEI and protamine are not adequate for the formation of the films. The approximate spherical shape of polymers assures a sufficient concentration of charge for their action as effective crosslinking agents among denaturated DNA strands.
- g) Films present supramolecular chirality. Circular dichroism studies show a very strong signal in a region of the spectrum that cannot be attributed to the double helix of DNA but, instead, to a

supramolecular arrangement of the molecules. Indeed, the slow cooling applied in film formation may provide time enough for the establishment of this arrangement. Here, the question is whether this supramolecular arrangement is due to an organization in the upper layer of the films (formed by aggregated spherical particles), in the lower layer of the films (the more compact zone) or in both.

As such, based on the above information, a model for film formation (Figure 87) is proposed that tries to explain what happened during the process.

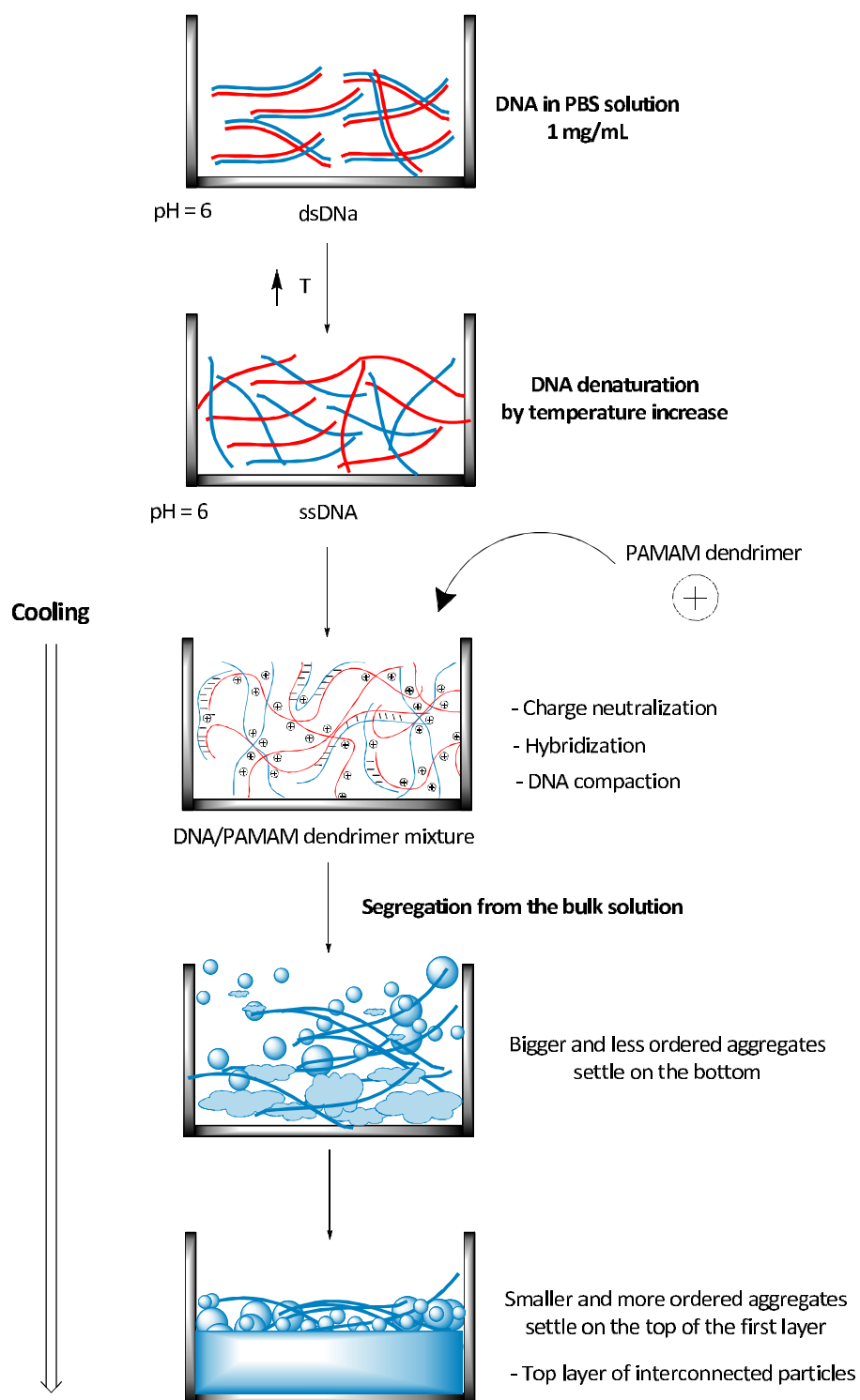


Figure 87 - Scheme with possible mechanism for the formation of DNA/ dendrimer film onto a surface.

So, in brief, we believe that, along film preparation, the following steps may occur:

1. SS-DNA is heated above its T_m and denatures in an important extent.
2. When immersed in the SS-DNA solution at a pH of 6, dendrimers become strongly protonated and immediately start to establish electrostatic interactions with the negatively charged phosphates groups in DNA.
3. When the cooling process starts, a random hybridization of the SS-DNA starts to occur simultaneously with SS-DNA compaction conducted by the dendrimers; dendrimers may even form "crosslinks" with different SS-DNA molecules. Also, supramolecular organization can take place.
4. Dendrimer addition also results in DNA charge neutralization. As such, neutral (or near neutral) aggregates form that tend to be segregated from the bulk solution.
5. Bigger SS-DNA/dendrimer aggregates, likely less ordered, have a higher rate of sedimentation and are deposited in the bottom of the flask forming a more compacted layer of material.
6. Smaller SS-DNA/dendrimer aggregates remain more time in the bulk solution, thus probably achieving a higher degree of internal organization. Possibly, there are chains of DNA that wrap around the dendrimers, forming superhelices, as reported in many works describing the three-dimensional structure of complexes formed between dendrimers and DNA^{559,560,603,604}. The existence of these superhelices may be responsible for the film chirality. As such, structures that are analogues of nucleosomes may form and, since they are linked, they form spherical arrays that finally lay down over the more compacted layer formed in the bottom of the flask.
7. The integrity and continuity of the film is assured by base pairing established between segments (tails) of single stranded DNA belonging to different molecules. These interactions may be established between DNA inside the same layer or inter-layers.
8. Since there is an excess of dendrimers in solution, these molecules adsorb to the surface of the film which become positively charged.

3.7. Cell culture studies using DNA/dendrimer-based films - improvement of cell adhesion

Having in view the possibility of using the DNA/dendrimer-based films in biomedical applications that require a close contact with cells, we first analysed cell adhesion over their surfaces. For these studies, films prepared with G3 PAMAM dendrimers, at a N/P ratio of 4, were prepared inside flasks containing pre-cleaned glass coverslips ($\varnothing = 1.3$ cm) in their bottom. Afterwards, the coverslips with the films were washed and transferred to 24-well plates ($\varnothing = 1.56$ cm). Each coverslip covered about 83 % of the well. NIH 3T3 cells were then seeded at a density of 4×10^4 cells/well and, 96 h later, the cultures were observed using an inverted microscope.

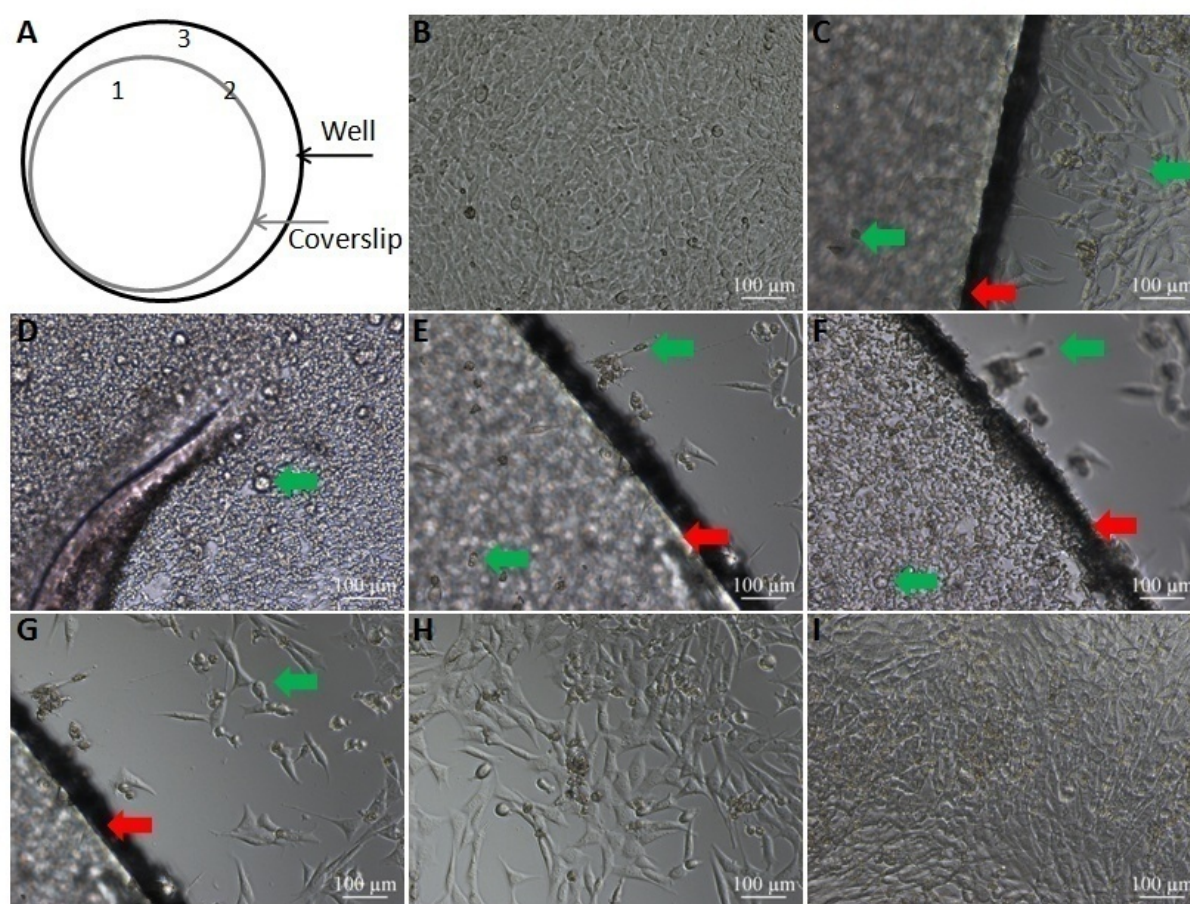


Figure 88 - Microscopic images of NIH 3T3 cells cultured on a well containing a DNA/G3 PAMAM dendrimer-based film (at a N/P ratio of 4), 96 h post-seeding. The red arrows indicate the film side, at the edge of the coverslip, while green arrows are pointing the cells. (A) Scheme of the coverslip with film inside the well, with zone 1 being the film, zone 2 the border between the film and the plastic surface of the well, and zone 3 the plastic surface of the well. (B) NIH 3T3 growing in the plastic surface (control). (C) Zone 2, with focus on adherent cells growing in the plastic. (D) Zone 1, with non-adherent cells. (E) Zone 2, with focus on adherent cells growing in the plastic. (F) Zone 2 (same as E), but with focus on the film. (G) Zone 3 (inner), near the film border, with focus on adherent cells growing in the plastic. (H) Zone 3 (middle), towards the well wall. (I) Zone 3 (outer), near the well wall. All images are at 100x magnification.

The results obtained are summarized in Figure 88. The number of cells that can be seen growing in the top of the films is very low. Here, most of the cells are rounded instead of presenting the typical fusiform shape of fibroblasts. However, the presence of the films did not impair cell growth in the surrounding plastic surface. Indeed, cell density near the film was lower and increased with the distance from to film (outwards from the film) (Figure 88, G-I). These preliminary results showed that the films did not allow for the cell adhesion but, nevertheless, they were not cytotoxic.

A second experiment was performed, where 5×10^4 cells were seeded on the top of the film in a small volume (a drop of cell suspension of about 30 μL), so that cells would not spread to the plastic surface of the well. After 3h, 6h, 24 h and 72 h of incubation, cell growth was evaluated using the inverted microscope (Figure 89).

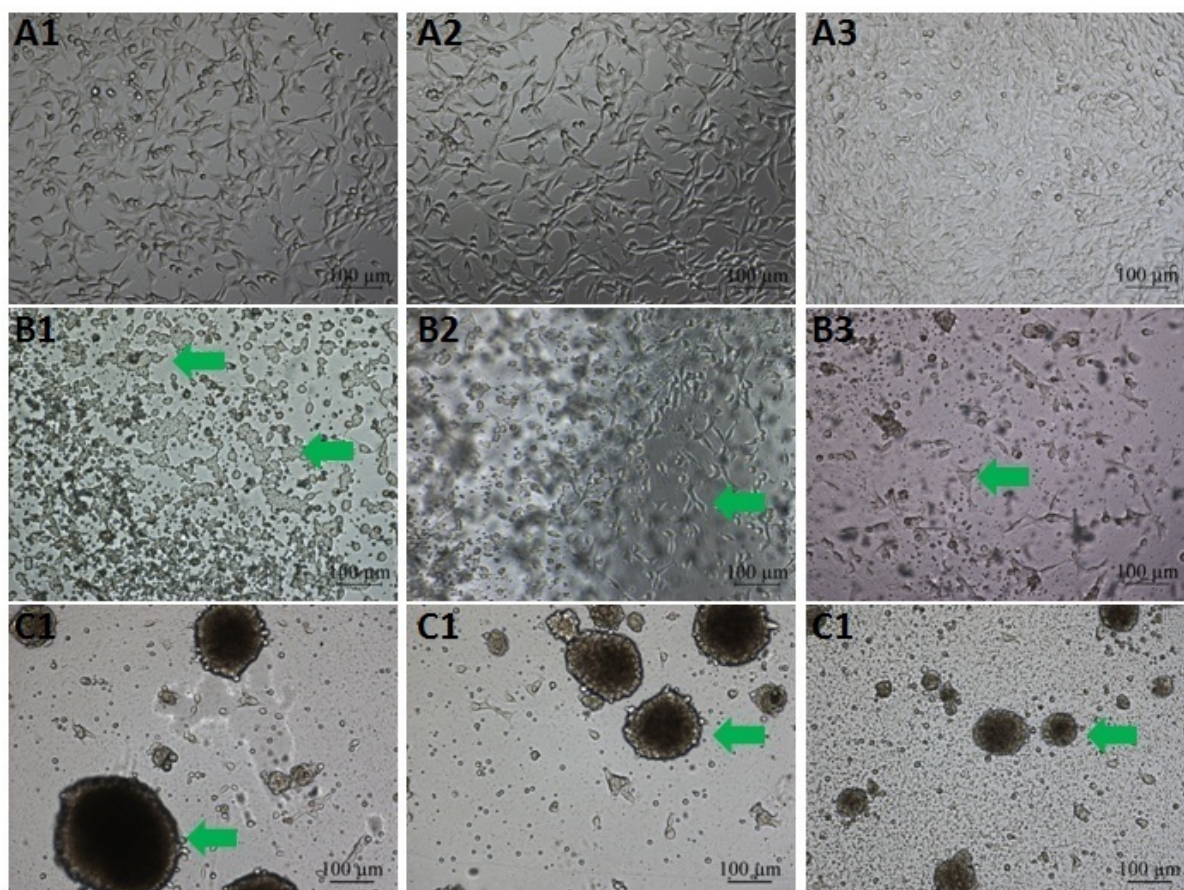


Figure 89 - Microscopic images of NIH 3T3 cells cultured on top of a DNA/G3 PAMAM dendrimer-based film (at a N/P ratio of 4). Cells on the plastic surface of the well (control) after (A1) 3 h, (A2) 6 h and (A3) 24 h. Cells on top of the film after (B1) 3 h, (B2) 6 h, (B3) 24 h and (C1) 72 h.

In this second experiment, after 3h of cell culture, it was possible to observe round shape cells on the top of the film, trying to adhere. When the incubation time increased to 6h, cells adopted the typical fusiform shape of fibroblasts. At 24 h, the fusiform shape of the cells was visible and more

pronounced, but the number of adherent cells decreased. At 72 h, almost no cells were visible attached to the surface and dark spheroid-shaped colonies appeared. As such, these results confirmed that the produced films were not suitable for cell adhesion. In fact, as previously shown, the surface of the films presents a very high positive zeta potential ($+24 \pm 5$ mV) which may be the reason why cell adhesion is so low. Cationic polymers are known to interact with the negatively charged cell membranes, causing damage and thus cytotoxicity⁵²⁷.

Based on the previous results, in order to improve cell adhesion, it was decided to functionalise the films with cyclic RGD (cRGD) peptides. RGD peptides (arginine-glycine-aspartic acid) are known to have a very important role in the extracellular matrix (ECM) as they are able to interact with integrin receptors existent in the surface of cells, supporting cell adhesion and proliferation⁶⁰⁵. RGDs have been explored in many published works to improved cell adhesion in a wide variety of substrates⁶⁰⁶ and to provide cell targeting capabilities to drug delivery systems⁵⁴⁸. The approach followed here was to prepare the DNA films and, afterwards, to modify their surface with the cRGD moieties by conjugation to the dendrimers already existing in the film (Figure 90). This approach was adopted, instead of functionalising the dendrimers prior to film preparation, as modifications in the dendrimers could impair film preparation. Also, being a peptide, RGD suffers denaturation at elevated temperatures, and could thus be damaged if subjected to 99°C (a condition for film formation)⁶⁰⁵.

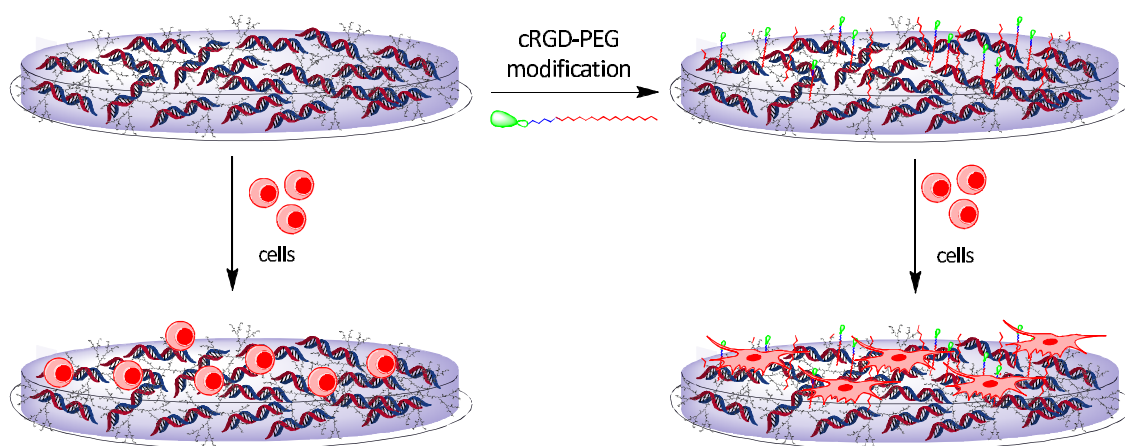


Figure 90 - Approach to improve cell adhesion by modifying previously prepared DNA films with cRGD peptides.

So, first, following the experimental methodology described by He *et al.*⁵⁴⁸, the cRGD peptides were conjugated to NH₂-PEG-COOH (spacer) via a thioether bond, using the heterobifunctional crosslinker EMCS, at a molar ratio of 1:1:1 to give cRGD-EMCS-NH-PEG-COOH, designated as cRGD-PEG (Figure 91).

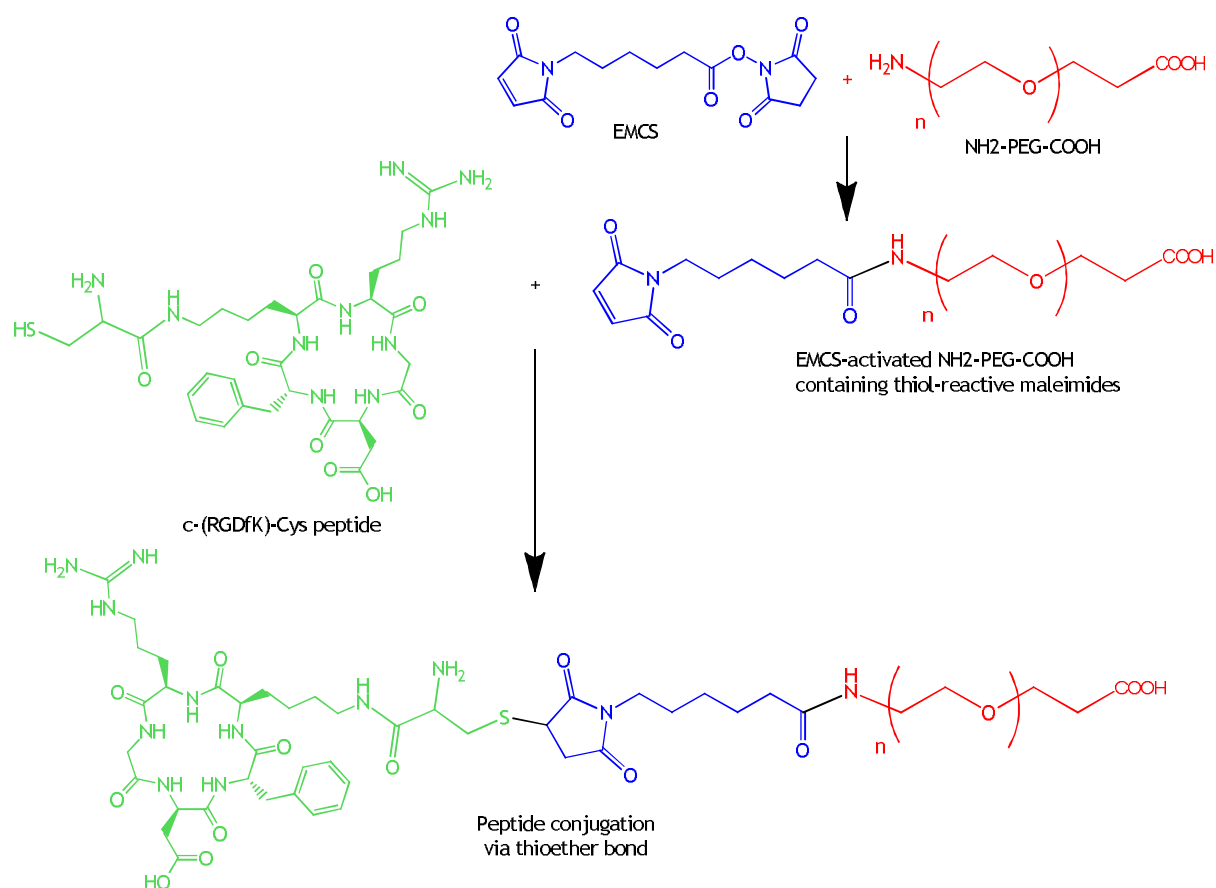


Figure 91 - Scheme for cRGD modification with NH₂-PEG-COOH via thioether bond, with the heterobifunctional crosslinker EMCS, resulting in cRGD-PEG.

The NMR spectra of the starting materials and final product are presented in Figure 92 and Figure 93, respectively.

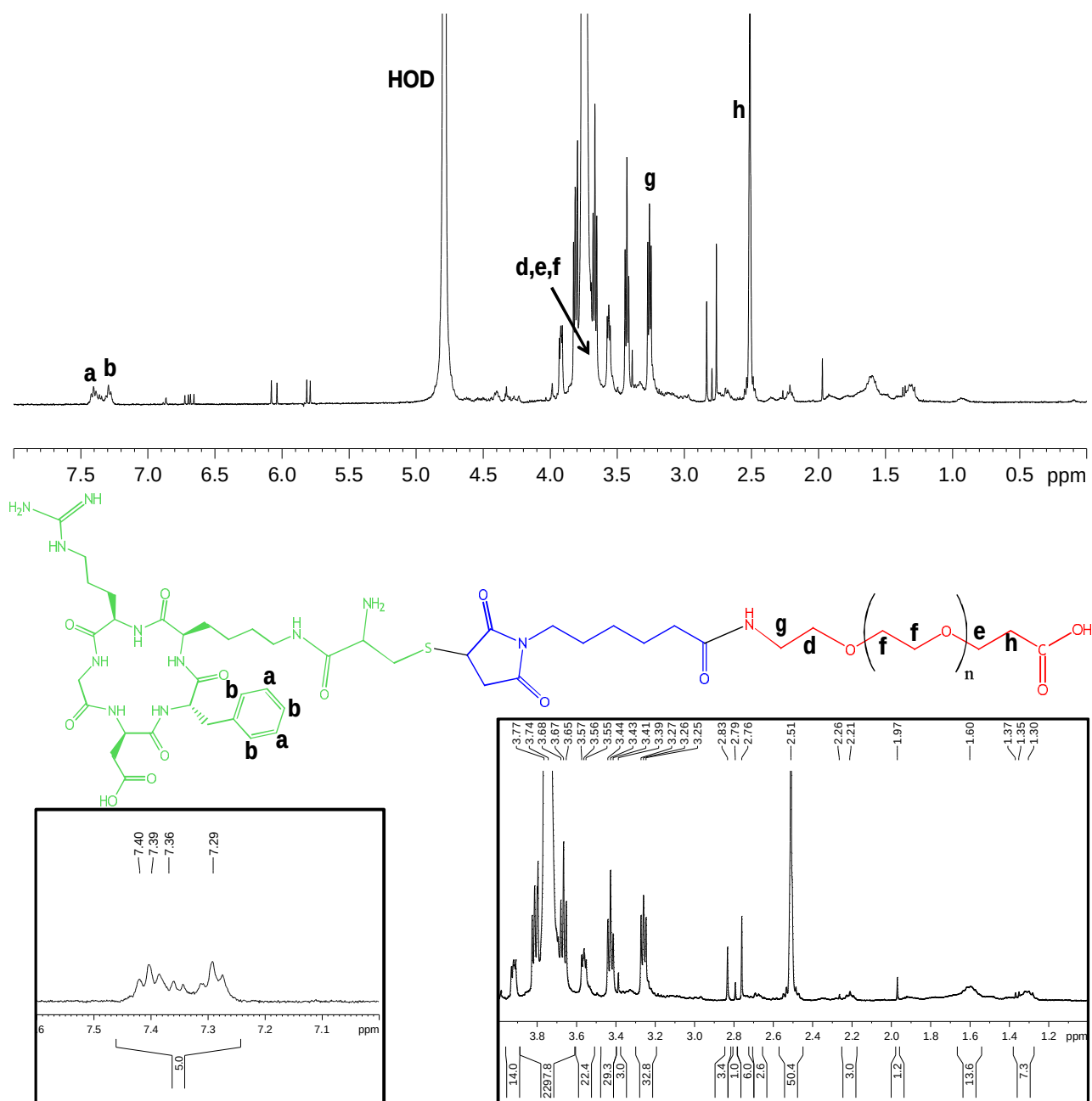


Figure 93 - ^1H NMR spectrum (in D_2O) of cRGD-PEG, with identification of the characteristic peaks of cRGD and $\text{NH}_2\text{-PEG-COOH}$ and corresponding integration.

According to Figure 93, the characteristic peaks of each starting material can be seen in the ^1H NMR spectrum of cRGD-PEG showing that conjugation was successful. There, it is possible to observe the PEG methylene protons ($-\text{OCH}_2\text{CH}_2-$, 182H, $\delta = 3.5 - 4$ ppm) and the cRGD peptide aromatic protons from the phenylalanine residue ($-\text{CH}$, 5H, $\delta = 7.3 - 7.5$ ppm), being this results in agreement with the expected values⁵⁴⁸.

Peak integration (Figure 93) showed that the achieved level of conjugation was 1 cRGD per every 12 $\text{NH}_2\text{-PEG-COOH}$ (*i.e.* 2298H : 182H per $\text{NH}_2\text{-PEG-COOH}$ = 12.6 $\text{NH}_2\text{-PEG-COOH}$). Indeed, the yield of this reaction is highly dependent on the stability of the intermediary products, as they might

suffer hydrolysis during the reaction. Also, as the molecular weight of both species are very close to each other, the dialysis membrane MWCO was not sufficient to separate them. Free cRGD and side products were removed by dialysis, but free $\text{NH}_2\text{-PEG-COOH}$ remained together with the conjugate. This means that both the conjugates and $\text{NH}_2\text{-PEG-COOH}$ will be used to functionalise the surface of the films. Although this seems a bad result, in fact it is not a problem since there is no need to cover all the surface of the films with RGDs. The idea is to endow the films with a sufficient number of RGDs to allow for the interaction with integrins at the cell surfaces (resulting in cell adhesion) but, on the other hand, it is also important to diminish the positive charge of the surface ($\text{ZP} = +24 \pm 5\text{mV}$) which can be achieved using a “PEG camouflage”. So, further functionalisation of the films with $\text{NH}_2\text{-PEG-COOH}$ will help to mask their positive surface charge and, possibly, increase their cytocompatibility.

DNA films based on G3 PAMAM dendrimers (at a N/P ratio of 4) were, then, prepared and modified at the surface with cRGD-PEG and $\text{NH}_2\text{-PEG-COOH}$ according to the reaction shown in Figure 94. This reaction was performed at a $[\text{cRGD-PEG} + \text{NH}_2\text{-PEG-COOH}] : [\text{initial G3 PAMAM dendrimers}]$ ratio of 1.4 : 1.

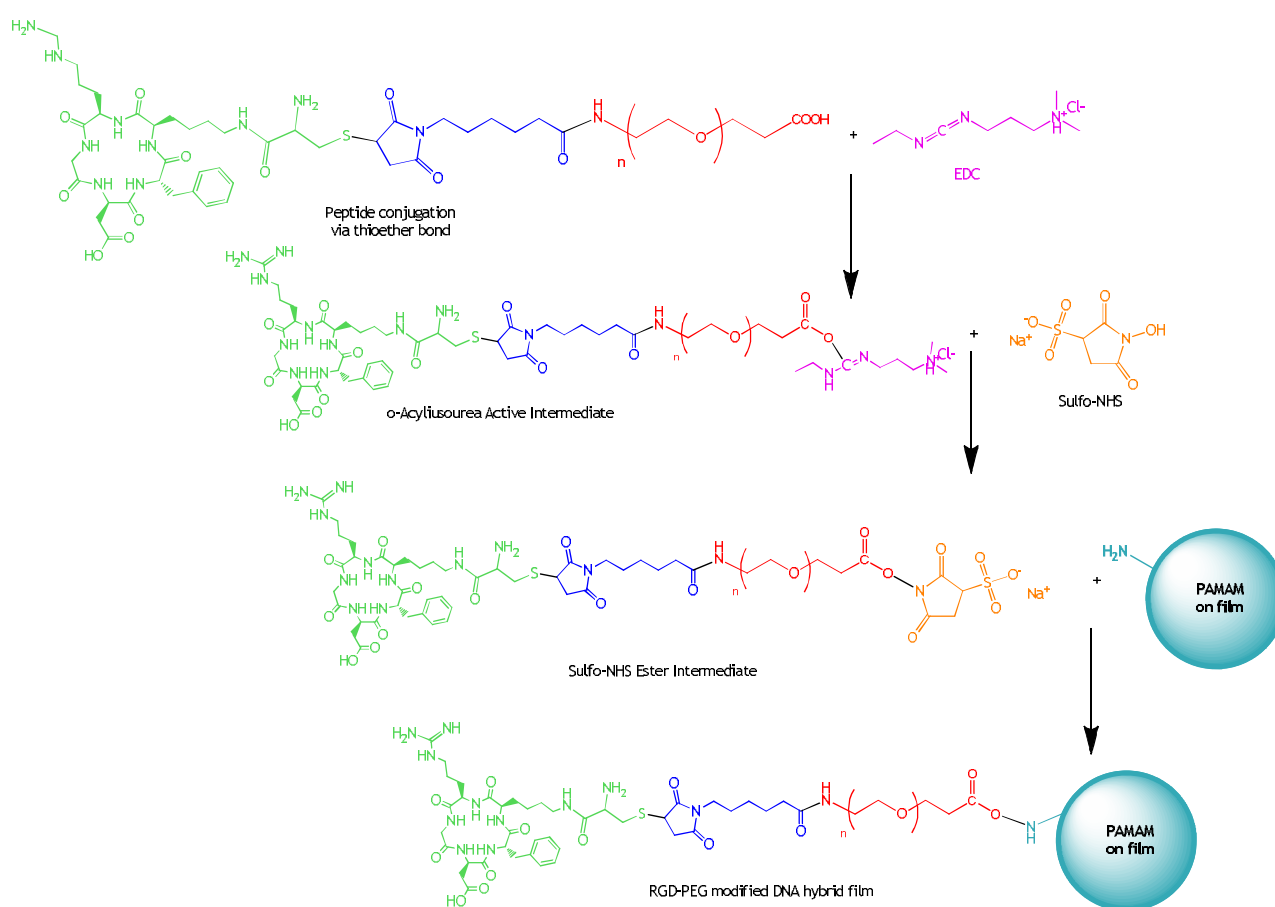


Figure 94 - Scheme of the reaction between cRGD-PEG molecules and PAMAM dendrimers existent at film surface. Functionalisation was done via EDC-sulfo-NHS carbodiimide reaction. The same reaction occurs with $\text{NH}_2\text{-PEG-COOH}$.

Attempts were made to quantify the extension of film functionalisation with cRGD-PEG. Since cRGD is a peptide, the bicinchoninic acid assay (BCA) for protein quantification was used. This is a colorimetric assay that is especially influenced by cysteine, tyrosine and tryptophan residues in proteins, being based on the Biuret reaction⁶⁰⁷. In this reaction, proteins reduce Cu^{2+} to Cu^+ in an alkaline medium and the solution becomes light blue. Cu^+ is then detected by the bicinchoninic acid forming a water-soluble complex that turns the solution bright purple, with absorbance at 562 nm.

A standard calibration curve was prepared for cRGD quantification using the BCA assay performed according to the manufacturer (in a microplate, 30 min at 37°C), with absorbance measurements at 550 nm. As a control, another one was also done using bovine serum albumin, BSA. Also, the possible interference of dendrimers and SS-DNA in the assay was studied by preparing solutions containing different concentrations of these molecules and applying the same procedure as for cRGD and BSA. The dendrimers produced a very low response in the assay and SS-DNA did not influence the assay at all (Figure 95).

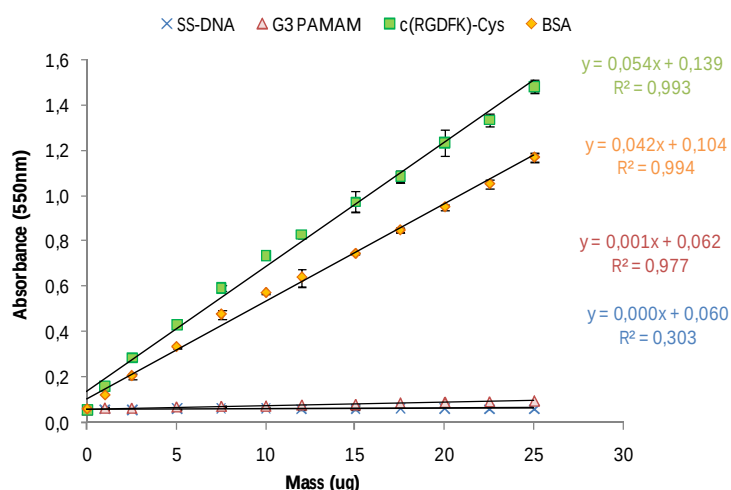


Figure 95 - BCA assay for protein quantification. Standard calibration curves for cRGD and BSA are shown, as well as the response of the assay to increasing concentrations of SS-DNA and G3 PAMAM dendrimers.

However, the application of the BCA assay for quantification of the RGD peptides present in the films was not feasible. Based on the results of our initial experiments, we already knew that direct quantification of immobilized cRGD-PEG in the DNA films could not be performed as the alkaline pH resulted in film dissolution. Indeed, this result was confirmed since the addition of the alkaline Cu^{2+} and BCA solution resulted in immediate dissolution of the film. Indirect quantification of RGDs in the film by measurement of their concentration in solution (free cRGD-PEG) after the conjugation process was also shown to be problematic. In the conjugation reaction, cRGD-PEG, imidazole, EDC and sulfo-NHS are present in solution. In fact, when the BCA assay was applied, an enhanced response was obtained due to sulfo-NHS. This finding was supported by the strange results

obtained using sulfo-NHS control solutions. In fact, it has been reported that sulfo-NHS is an interferent in this BCA assay, leading to inaccurate protein quantification⁶⁰⁸. On the other hand, we raised the hypothesis of quantifying free cRGD-PEG by UV-vis spectroscopy based on the typical protein absorbance at 280 nm, but this was also not possible, since EDC, sulfo-NHS and imidazole have maximum absorbances at 214 - 225 nm, 260 - 280 nm and 280 nm, respectively.

Nevertheless, the effect of RGDs' presence on cell adhesion and proliferation was evaluated, as a successful conjugation would result in an increase in cell adhesion and viability when compared with native DNA films. For these studies, native and cRGD-PEG functionalised SS-DNA films were used based on G3 PAMAM dendrimers at a N/P ratio of 4. NIH 3T3 fibroblasts were then cultured on both types of films, and cultures were observed by optical microscopy after 24 h and 48 h of cell seeding (Figure 96). Results showed that, after functionalisation with cRGD-PEG, the number of cells present on the top of the films increased. Cells also seemed to be more adherent to the films, presenting the typical elongated fibroblast morphology.

Cell viability/growth was also compared for native and functionalised films based on a metabolic activity assay (rezasurin reduction assay). The quantitative results shown in Figure 97 (A) revealed that films' functionalisation with cRGD-PEG was beneficial for cell adhesion and proliferation. The adhesion and growth of the cells cultured on the plastic surface was, of course, always higher (Figure 97, B) but one should have in mind that these surfaces are especially treated to increase cell adherence.

In conclusion, although it was not possible to quantify the extension of films' functionalisation, the present results are very interesting as a proof-of-concept, since they clearly reveal that cRGD-PEG conjugation to SS-DNA films increase cell adhesion. In this sense, these systems deserve to be further and deeper explored in the future, namely aiming at tuning their cell adhesion properties through a more accurate control of functionalisation with cRGD-PEG.

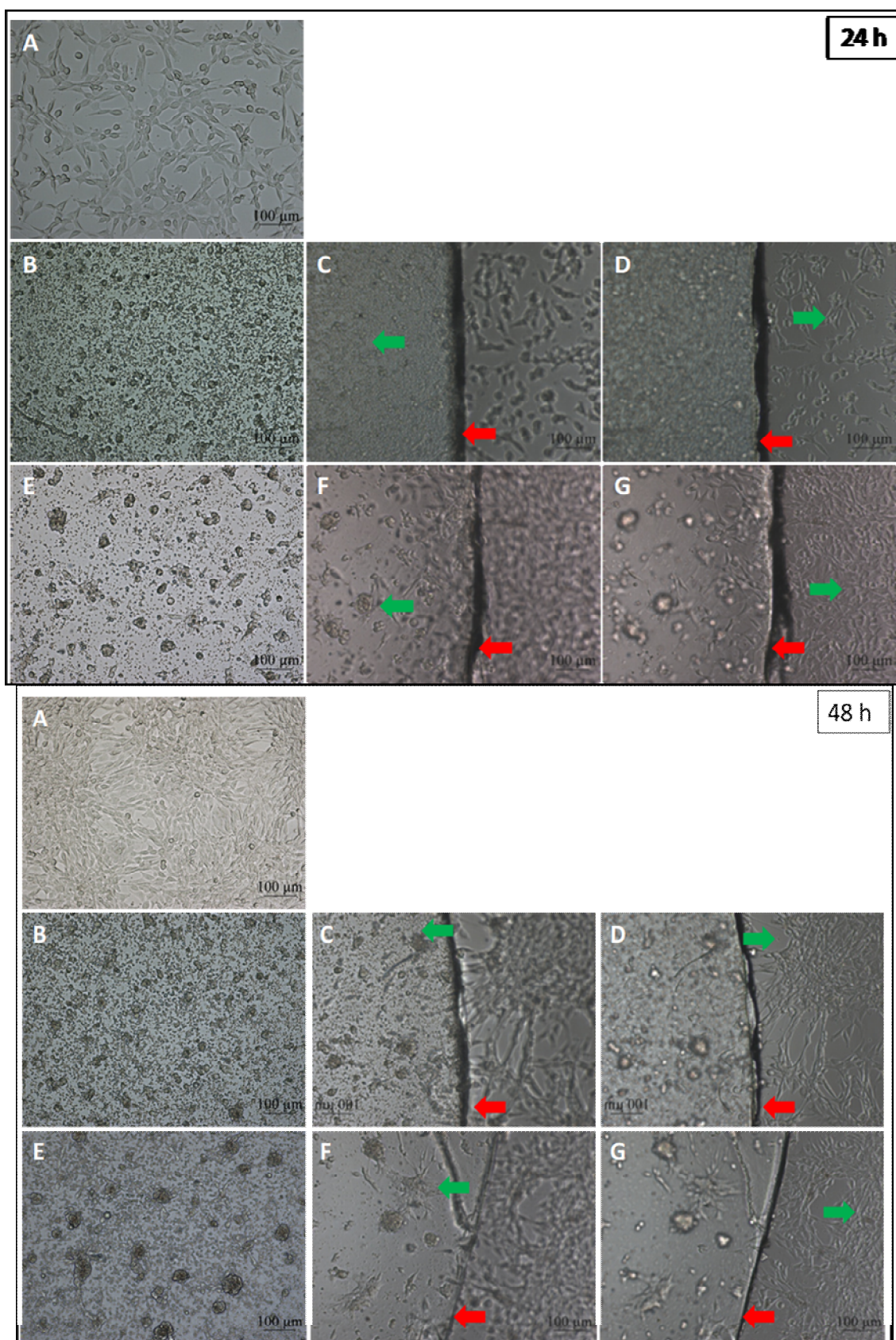


Figure 96 - Microscopic images of NIH 3T3 cells after being culture for 24 h and 48 h on the top of native films and films functionalised with cRGD-PEG. The red arrows indicate the film side (at film and well border), while the green arrows indicate the focus side. (A) Cells cultured on the plastic surface of the well (control), (B, C, D) cells cultured on native DNA/G3 PAMAM films, and (E, F, G) cells cultured on cRGD-PEG functionalised films. Scale at 100 µm.

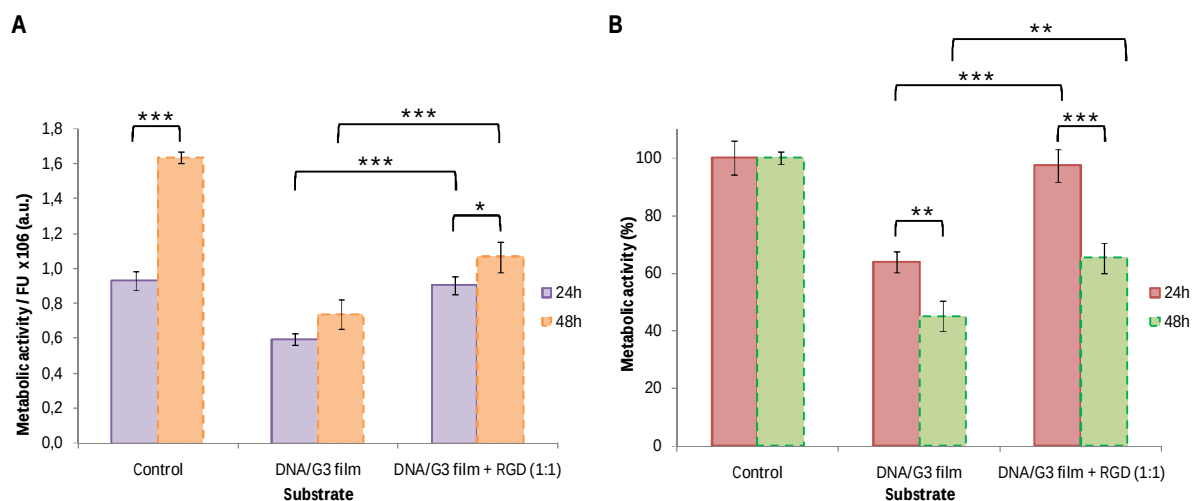


Figure 97 - NIH 3T3 cell viability/growth when cultured on the top of native films and films functionalised with cRGD-PEG (as assessed by a metabolic activity assay). SS-DNA films were used based on G3 PAMAM dendrimers at a N/P ratio of 4. Results are shown after 24 h and 48 h after cell seeding. (A) Metabolic activity expressed as arbitrary fluorescence units and (B) metabolic activity expressed as a percentage relative to the control (cells cultured on the plastic surface of the wells). Results are represented as mean $\pm$ s.d., with $n = 4$. Statistical analysis shows $p < 0.03$ (*), $p < 0.0002$ (**), $p < 0.0001$ (***)

3.8. Evaluation of DNA/dendrimer-based films as drug delivery systems

3.8.1. DNA/dendrimer-based films drug loading

As seen in the first chapter, DNA materials, such as hydrogels and films, among many other applications, have been studied as drug delivery platforms and environmental pollutant removal systems. This is possible since there are several molecules that possess a high affinity to bind DNA. For example, several chemotherapeutic drugs are efficient in anticancer therapy due to their ability to bind DNA, thus inhibiting cell division and proliferation⁶⁰⁹. Based on this information, we decided to test the behaviour of the DNA/dendrimer-based films as drug delivery systems. For these studies, three anticancer drugs were chosen based on their properties, namely doxorubicin (DOX, a DNA intercalator), cisplatin (cisPt, which can establish covalent bonds to DNA) and 5-fluorouracil (5-FU, which does not have particular propensity to bind DNA).

Indeed, there are several types of chemotherapeutic drugs, like alkylating agents (those that directly damage DNA), antimetabolites (those that substitute the normal building blocks in RNA and DNA) and antibiotics (those that interfere with enzymes in the DNA replication process)⁶⁰⁹. The antineoplastic drugs DOX and cisPt are well known drugs in the field and are often used as comparative standards^{609,610}. The mechanism of action of DOX (antibiotic) is either by inhibiting

topoisomerases I and II (responsible for separating the two DNA strands prior replication) and/or by intercalating directly in the DNA's double helix⁶⁰⁹ (the DOX tetracene ring system is inserted between the planar base pairs of DNA and the daunosamine sugar moiety occupies the minor groove)⁶¹¹, inhibiting the progression of DNA and RNA polymerases⁶⁰⁹. On the other hand, cisPt (alkylating agent) enters the cell, suffers aquation (the chlorides are replaced by water molecules) and binds to DNA bases, originating DNA adducts (intramolecularly, more than 90% of the times) or DNA crosslinks (intermolecularly, less than 2% of the times)^{610,612}. Both these antineoplastic drugs interfere with DNA replication. Because DNA repair mechanisms are not successful, apoptosis is triggered and cell proliferation decreases. The molecule 5-FU is also an antineoplastic drug (antimetabolite), which mechanism of action is believed to be through the binding of its metabolites (FdUMP, FdUTP, FUTP) to thymidylate synthase (TS) and a folate cofactor, forming a stable ternary complex. This inhibits TS activity, essential for DNA replication and repair⁶¹³. Also these antimetabolites can be incorporated into DNA and RNA, unbalancing the misincorporation, excision and repair cycles, resulting in DNA breaks and cell death and interference in protein synthesis.

To evaluate whether DNA films were able to be loaded with these drugs, SS-DNA films based on G3 PAMAM dendrimers (at a N/P ratio of 4) were prepared as usual, washed with PBS and incubated with the different anticancer drugs, at different concentrations (all drug solutions were prepared in PBS). The DNA/dendrimer-based films were incubated with the different drug solutions for 24 h, 48 h and 72 h, and then the amount of loaded drug was indirectly quantified by measuring the amount of drug that remained in solution. Free DOX and 5-FU in solution were quantified by their spectroscopic properties (DOX is a molecule that emits fluorescence at 590 nm and 5-FU absorbs light at 266 nm). Regarding cisPt, as this compound has a low molar absorptivity in the UV region and no fluorescence⁵⁴⁹, selective derivatization was needed for its quantification (Figure 98). Standard calibration curves were prepared for drug quantification purposes.

As expected, all 5-FU was detected free in solution, revealing that no 5-FU did not interact with the DNA in the film nor was loaded into the films (data not presented). Regarding DOX and cisPt, the amount of loaded drug was higher for DOX than for cisPt (Figure 99). Also, another contribution for lower cisPt loading may be the solution used for incubation. All drugs were prepared in PBS at the desired concentrations. For cisPt this is especially important since in these conditions it will not suffer aquation. According to the literature⁶¹⁰, in isotonic saline solution (mimicked by PBS) or extracellular fluid, where the chloride concentration is ≈ 150 mM, cisPt does not suffer aquation. On the other hand, in the intracellular compartment where chloride concentration is much lower (≈ 7 mM)⁶¹⁴, cisPt aquation is favoured and it will bind DNA.

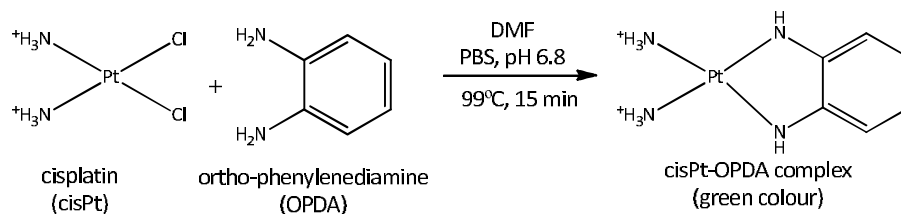


Figure 98 - Cisplatin derivatization with OPDA gives a green colored complex (cisPt-OPDA) with maximum absorbance at 706 nm.

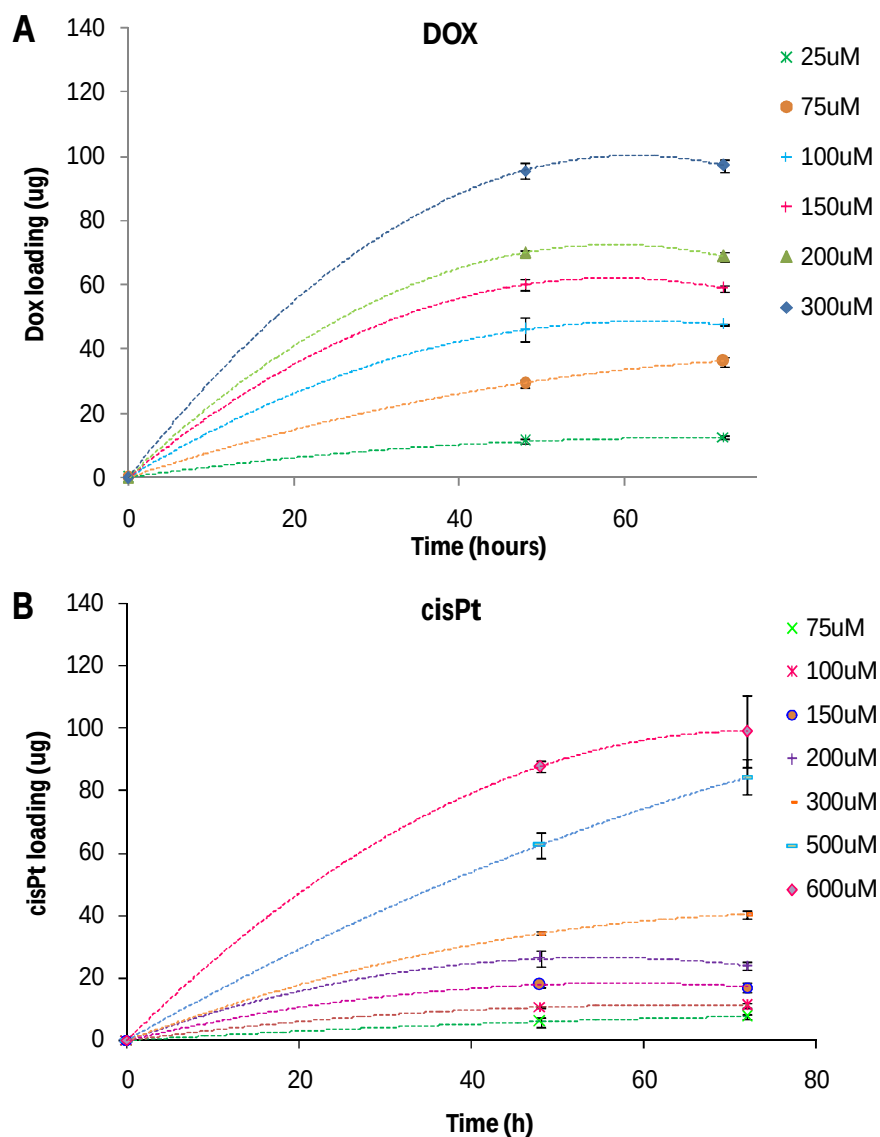


Figure 99 - Drug loading profiles for SS-DNA films based on G3 PAMAM dendrimers (at a N/P ratio of 4) upon increasing incubation times and using solutions containing different drug concentrations. (A) DOX loading profile. (B) cisPt loading profile. Results are represented as mean $\pm$ s.d., with $n = 5$.

In Figure 99, it is possible to observe that the extent of drug loading depends on the concentration of the drug solution used in the process. This is true for both DOX and cisPt and

reveals the high capacity of the films to accommodate these drugs in their structure. Also, independently of the drug and drug concentration, the amount of loaded drug always reaches a plateau after a certain incubation period, which should be related with an equilibrium established between the drug in the film and the drug in solution. Overall, after 72 h incubation with DOX solutions of increasing concentration, the percentages of drug loading in the DNA films were: 93.5 % (25 μ M), 88.6 % (75 μ M), 87.9 % (100 μ M), 72.3 % (150 μ M), 63.4 % (200 μ M) and 59.6 % (300 μ M). For the same time period and for cisPt, the percentages of drug loading in the DNA films were: 32.9 % (75 μ M), 37.0 % (100 μ M), 37.7 % (150 μ M), 40.0 % (200 μ M), 44.9 % (300 μ M), 56.2 % (500 μ M) and 55.1% (600 μ M).

3.8.2. DNA/dendrimer-based films drug release

After drug loading studies, experiments were done to study the drug release profile for both DOX and cisPt. As such, drug-loaded DNA films were exposed to different solutions mimicking the fluids in the extracellular environment, and the released drug was quantified over time. The extent of DOX release was accessed by fluorescence measurement at $\lambda_{em} = 590$ nm ($\lambda_{ex} = 530$ nm), whereas the amount of released cisPt was based on the absorbance of cisPt-OPDA at 706 nm. The studies were done with several films prepared from SS-DNA and G3, G4 and G5 PAMAM dendrimers, as well as from SS-DNA and brPEI-25, all at a N/P ratio of 4. The initial amount of drug present in the films was always determined using the methodology followed in the previous loading studies. These results are shown in Table 5 for DOX and in Table 6 for cisPt. The average DOX loading efficiency was $(98.1 \pm 0.2)\%$, $(97.90 \pm 0.03)\%$, $(97.95 \pm 0.06)\%$, and $(98.3 \pm 0.1)\%$ for, respectively, the films based on DNA/G3, DNA/G4, DNA/G5 and DNA/brPEI-25. Also, the average cisPt loading efficiency was $(58 \pm 8)\%$, $(62 \pm 1)\%$, $(62 \pm 2)\%$, and $(56 \pm 4)\%$ for, respectively, the films based on DNA/G3, DNA/G4, DNA/G5 and DNA/brPEI-25.

The loading efficiency of DOX was not significantly different for the different DNA films (Table 5), that is, did not change with the type of cationic polymer (dendrimer or brPEI-25). This observation shows that DOX interaction with films is predominantly through the SS-DNA. Note that the loading of these films was performed in slightly different conditions than those used in the previous experiments (the initial DOX concentration was 50 μ g/mL (≈ 92 mM) and 93 h of incubation time was used) and that explains why loading efficiency attained higher values. The same behaviour was observed when using cisPt which also possess a high tendency to bind DNA. In this case, drug loading was done using 1 mL of 50 μ g/mL (≈ 166 mM) cisPt in PBS and an incubation time of 72 h.

Table 5 - DOX loading efficiency (%) determined in the films used to study drug release profiles. The films were prepared from SS-DNA and G3, G4 and G5 PAMAM dendrimers, as well as from SS-DNA and brPEI-25, all at a N/P ratio of 4. DOX loading was done using 1 mL of 50 µg/mL ($\approx$ 92 mM) DOX in PBS and an incubation time of 93 h. Results are represented as mean $\pm$ s.d, with n = 30.

		DOX loading efficiency (%)
DNA/G3	Mean $\pm$ s.d.	98.1 $\pm$ 0.2
DNA/G4	Mean $\pm$ s.d.	97.90 $\pm$ 0.03
DNA/G5	Mean $\pm$ s.d.	97.95 $\pm$ 0.06
DNA/PEI	Mean $\pm$ s.d.	98.3 $\pm$ 0.1

Table 6 - CisPt loading efficiency (%) determined in the films used to study drug release profiles. The films were prepared from SS-DNA and G3, G4 and G5 PAMAM dendrimers, as well as from SS-DNA and brPEI-25, all at a N/P ratio of 4. DOX loading was done using 1 mL of 50 µg/mL ($\approx$ 200 mM) cisPt in PBS and an incubation time of 72 h. Results are represented as mean $\pm$ s.d, with n = 30.

		cisPt loading efficiency (%)
DNA/G3	Mean $\pm$ s.d.	58 $\pm$ 8
DNA/G4	Mean $\pm$ s.d.	62 $\pm$ 1
DNA/G5	Mean $\pm$ s.d.	62 $\pm$ 2
DNA/PEI	Mean $\pm$ s.d.	56 $\pm$ 4

Figure 100 shows the release profiles for doxorubicin (cumulative release) in different media, namely in H₂O, PBS solution, DMEM, 1% (v/v) FBS, 10% (v/v) FBS and complete DMEM (DMEM comp., that is, DMEM containing 10% (v/v) FBS and 1% (v/v) antibiotic-antimycotic solution). Drug release rate was higher in the initial times of the experiments and decreased afterwards. In most experiments, after approximately 3 days, the cumulative release attained a plateau. Interestingly, independently of the type of polymer/dendrimer generation used to prepare the films, DOX cumulative release in 10% (v/v) FBS and complete DMEM was always higher than in the other solutions. In these two conditions and for the films based on dendrimers, cumulative release was still increasing after 17 days, revealing a sustained release behaviour. The same was also verified by Yu *et*

a.⁶¹⁵, with serum-stable aptamers, loaded with DOX. Since complete DMEM also has 10% FBS, this increased release was attributed to the presence of FBS. The use of FBS in the test solutions is important since it closely mimics the physiological conditions. One should also notice that, for certain time points, the cumulative release values seem to decrease which is a strange situation. A possible explanation for this could be a partial adsorption of DOX to the microcentrifuge tubes (it is known that DOX stains labware) or the formation of DOX dimers in solution, due to the increase in its concentration, that result in fluorescence quenching (self-quenching).

The drug release results obtained for cisPt were disappointing since, for most conditions, no cisPt was detected in solution (for this reason, drug release profiles are not presented). Indeed, it is known that, in the case of DOX, the binding to DNA is reversible (interactions between DOX and DNA are non-covalent)⁶¹¹, while in the case of cisPt, covalent bonds are established with DNA making binding irreversible⁶¹⁶. In fact, after 72 h (Figure 101), cisPt presence was only detected for the solutions 1% (v/v) FBS and 10% (v/v) FBS. Again here, as observed in the case of DOX, FBS seems to increase drug release. However, unexpectedly, cisPt release was undetectable in complete DMEM (which also contains FBS).

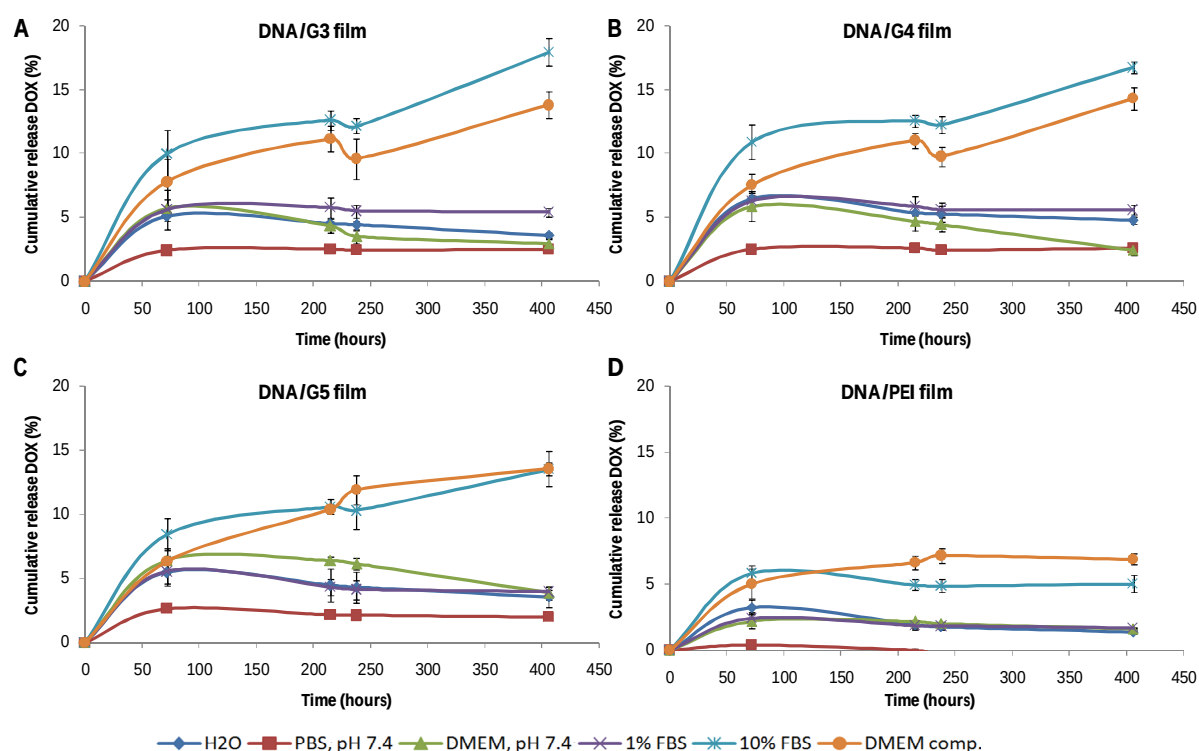


Figure 100 - DOX release profiles using DNA films prepared with different polymers, at a N/P ratio of 4, after immersion in solutions mimicking the fluids in the extracellular environment. Results are represented as mean $\pm$ s.d., with $n = 5$.

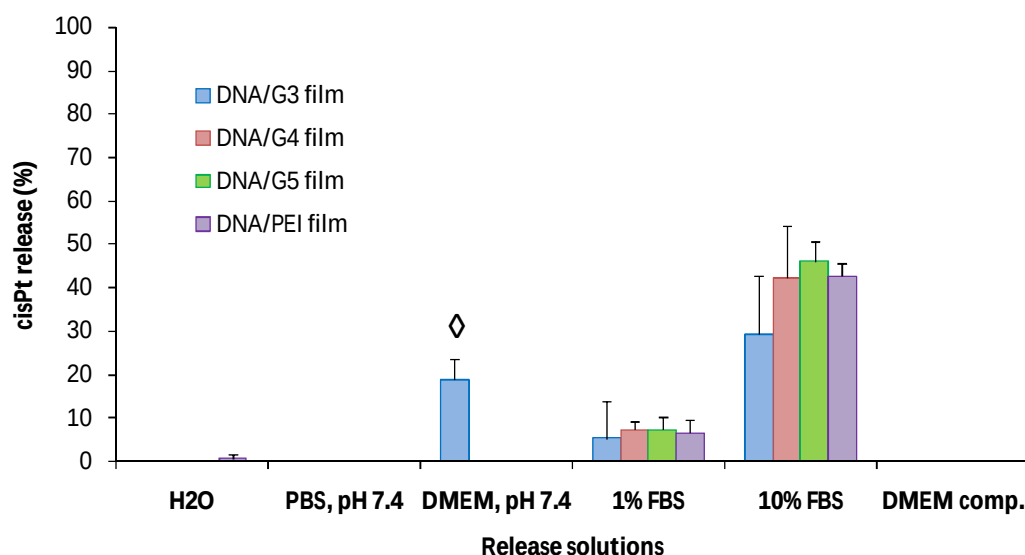


Figure 101 - cisPt release (%) from DNA films prepared with different polymers, at a N/P ratio of 4, after exposure to several solutions for 72 h. cisPt presence in solution was not detectable in most of the experiments. ♦ This result was discarded. Results are represented as mean $\pm$ s.d., with $n = 5$.

3.8.3. Use of DNA/dendrimer-based films as drug delivery platforms: cytotoxicity studies

The DNA films were evaluated as drug delivery systems using cells cultured *in vitro*. In these experiments, DOX-loaded and cisPt-loaded films were immobilized in the bottom of cell culture wells, while cells were cultured in the membrane of well inserts. So, the cells were not directly in contact with the films but only with the solution containing their release products (drug). For these studies, two cell lines were chosen, namely the ovarian carcinoma cell lines A2780 and A2780cisR, the last one resistant to cisplatin.

As a first step, the IC_{50} values for DOX and cisPt were determined using the two cell lines and the rezasurin reduction metabolic assay. The IC_{50} value corresponds to the drug concentration that inhibits 50% of cell growth which, in this case, is indirectly determined by measuring cellular metabolic activity. The IC_{50} value usually changes with the time of drug exposure and, so, experiments were performed exposing the cells to the drugs for 24 h, 48 h and 72 h. Cells were exposed to several drug concentrations, namely, 0-0.5 μ M DOX and 0-10 μ M cisPt for A2780 cells, and 0-1 μ M DOX and 0-100 μ M cisPt for A2780cisR cells. The results obtained in these experiments are shown in Figure 102 (as a percentage of metabolic activity in relation to cells non-exposed to the drug (control)) and Figure 103 (plots for determination of the IC_{50} values). As expected, cell metabolic activity was concentration and time dependent and A2780 cells were more sensitive to cisPt than A2780cisR cells. Similar results were observed when DOX was used. For both DOX and cisPt, the IC_{50} values were always higher for A2780cisR cells than for A2780cells, decreasing with the time of drug exposure.

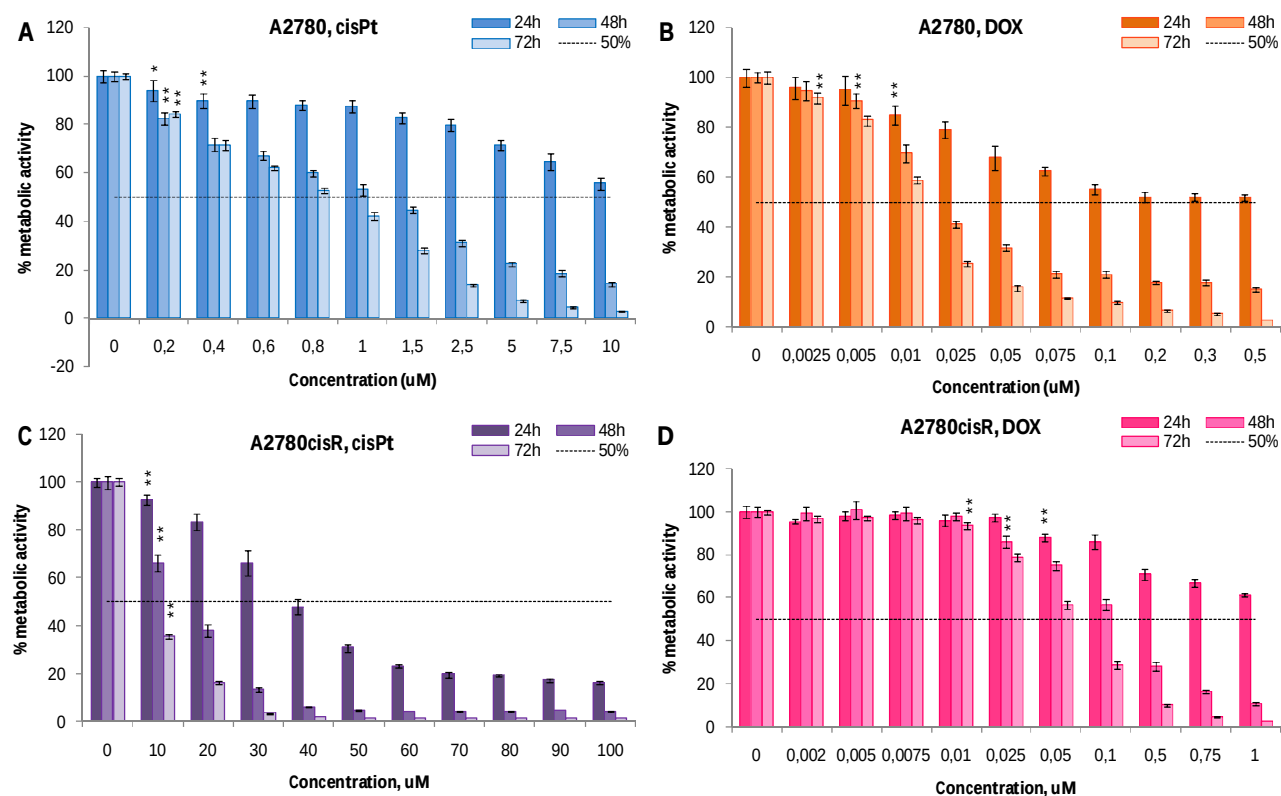


Figure 102 – Dose-response (metabolic activity) profiles for cells exposed to different concentrations of DOX and cisPt. A2780 cells were exposed to (A) 0-10 μ M cisPt and (B) 0 - 0.5 μ M DOX. A2780cisR cells were exposed to (C) 0 - 100 μ M cisPt and (D) 0 - 1 μ M DOX. Results are represented as mean $\pm$ s.d., with $n = 6$, expressed as a percentage in relation to the control. Statistical analysis compares the different concentrations with the control (0 μ M), at the corresponding time point. $p < 0.0002$ (*), $p < 0.0001$ (**) indicated at the first concentration with significant difference in relation to the control.

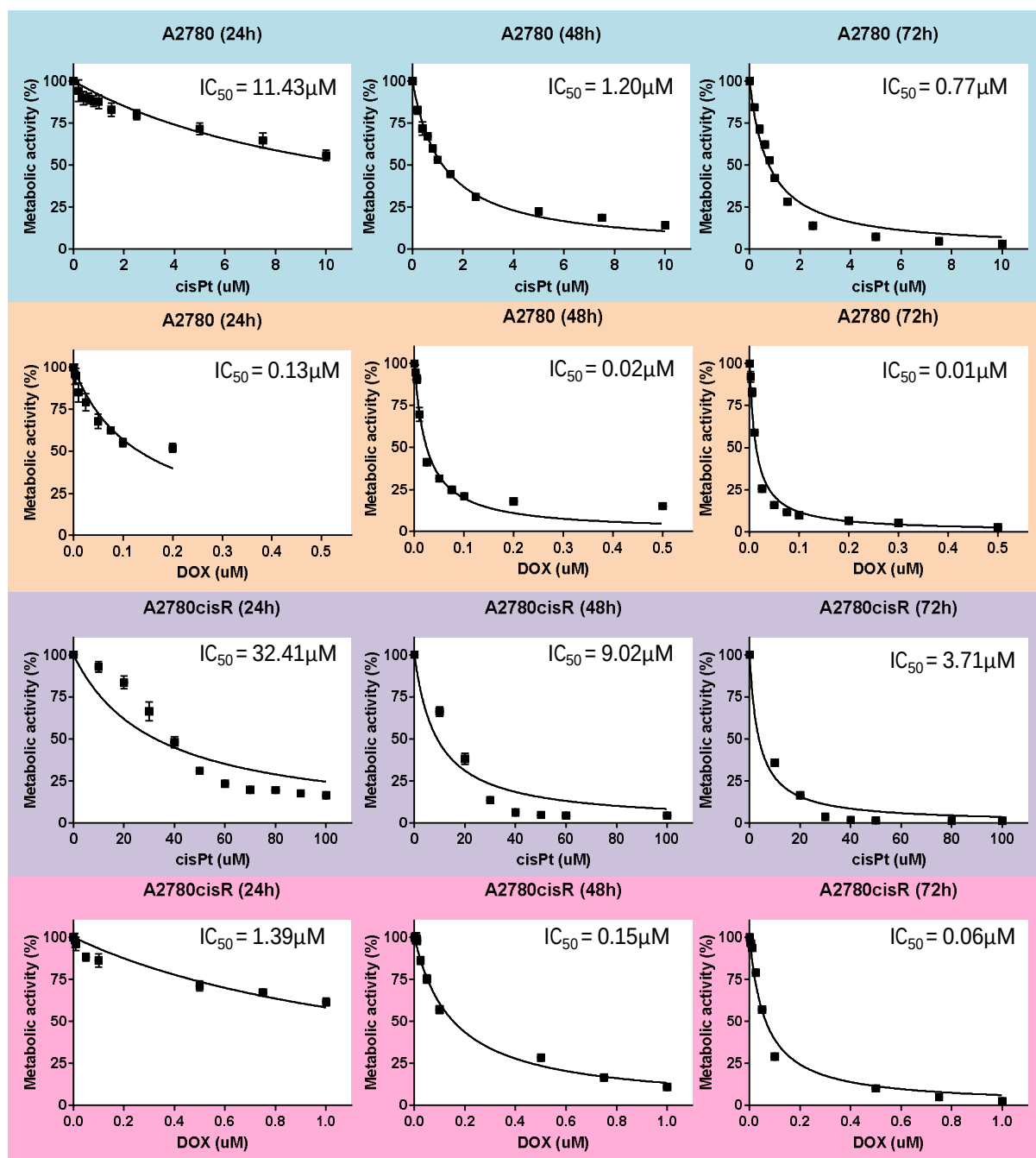


Figure 103 – Plots used for IC_{50} determination.

In order to evaluate the behaviour of the DNA films as drug delivery platforms, several films were prepared based on SS-DNA and G5 PAMAM dendrimers, and SS-DNA and brPEI-25, all at a N/P ratio of 4. Afterwards, the films were incubated with 1 mL of 50 $\mu g/mL$ DOX, 50 $\mu g/mL$ cisPt and 50 μg DOX + 50 μg cisPt, for 72 h. The DNA and drug contents in these films were first quantified (Table 7). G5 PAMAM dendrimers and brPEI-25 were chosen for film preparation since, theoretically, they are very close to each other in molecular weight (≈ 26 kDa and 25kDa, respectively) and number of amines (128 $-NH_2$ and ≈ 200 $-NH_2$, respectively).

Table 7 - DNA and drug content in films prepared for cytotoxicity experiments. Quantification was performed by UV-visible spectroscopy at 260 nm, 480 nm and 706 nm, for DNA, DOX and cisPt-OPDA, respectively. Results are represented as mean $\pm$ s.d., with n = 3.

		G5	G5 DOX	G5 cisPt	G5 DOX ¹ + cisPt ²	PEI	PEI DOX	PEI cisPt	PEI DOX ¹ + cisPt ²
DNA content	Mean	98.8	98.6	98.4	98.8	99.1	99.0	98.8	98.8
	s.d.	0.4	0.5	0.3	0.3	0.2	0.3	0.4	0.4
Drug content	Mean	-	78	52	78 ¹ 53 ²	-	83	52	90 ¹ 56 ²
	s.d.	-	8	1	5 ¹ 2 ²	-	5	2	8 ¹ 1 ²

Figure 104 shows the metabolic activity of A2780 and A2780cisR cells after 72 h-exposure to drug loaded DNA films (results are expressed as a percentage of metabolic activity in relation to the control, that is, cells cultured in the transwell in the presence of a non-loaded film).

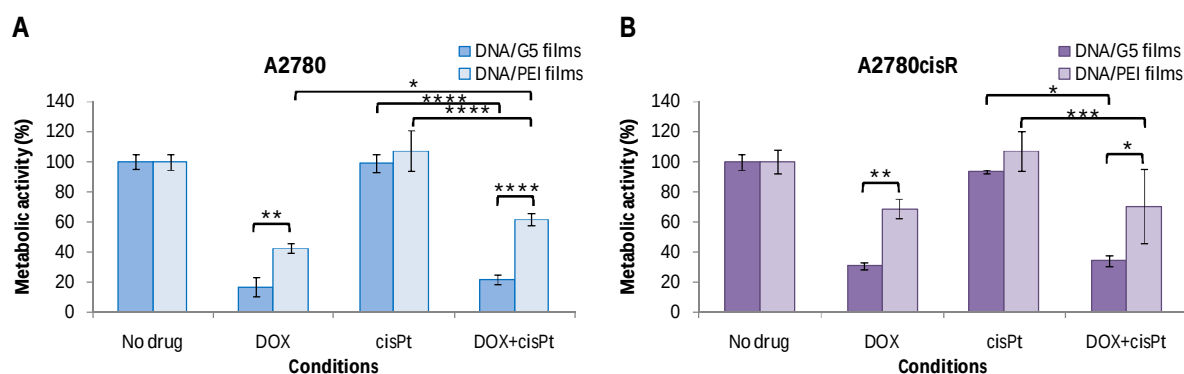


Figure 104 - Metabolic activity of A2780 and A2780cisR cells after 72 h-exposure to drug loaded DNA films. Results are represented as mean $\pm$ s.d., with n = 3, expressed as percentage of metabolic activity in relation to the control, that is, cells cultured in the transwell in the presence of a non-loaded film). Statistical analysis shows $p < 0.03$ (*), $p < 0.002$ (**), $p < 0.0002$ (***) and $p < 0.0001$ (****)

As expected, cell death only occurred in the presence of DOX-loaded films. These results are in accordance with the drug release data obtained in the previous section which revealed the impossibility of cisPt to be released from the films in complete DMEM. Also, when DOX was mixed with cisPt in the same films, the cytotoxicity results were not significantly different from those using DOX alone. In other words, cytotoxicity is attributed to DOX. Interestingly, when using DNA/ brPEI-25-based films, the cytotoxicity was always lower than when using DNA/dendrimer-based films.

These results were also expected since the extent of DOX release was also lower for DNA/ brPEI-25-based films than for DNA/dendrimer-based ones. Overall, results show that, among the drugs studied, only doxorubicin can effectively be released and thus exert its cytotoxic effects when using the prepared DNA films. Also, as seen before, A2780 cells were more sensitive to DOX than A2780cisR cells.

4. Conclusion and future perspectives

In this PhD work, novel DNA/dendrimer-based films representing a new class of materials were prepared. An innovative approach was used for film fabrication, by heating the DNA above its T_m , adding the dendrimers, and then cooling down the system very slowly. Ionic electrostatic interactions between PAMAM dendrimers and DNA, as well as hydrogen bond formation between complementary DNA strands, were the driving force for film formation.

To our knowledge, the method developed in this work in which a film composed of PAMAM dendrimers and DNA was prepared has not yet been described in the literature. Although our initial goal was to obtain a DNA/PAMAM dendrimer hydrogel, the experimental conditions used in the study (e.g., DNA concentration) were not conducive to the formation of a tridimensional network typical of hydrogels. Instead, a thin film was obtained. During the optimization process, it was possible to conclude that film preparation was dependent on many experimental conditions/variables. As such, after a set of optimization experiments, a general protocol for their fabrication was devised.

The information gathered throughout the optimization and characterization process was important to understand the molecular interactions occurring among the film components and to propose a theoretical model for film formation. The optimization process revealed that obtaining the films was dependent on:

- maximum temperature applied: fully denatured DNA is obligatory for film formation to occur and can only be obtained by heating the DNA solution above its melting temperature. In the case of temperatures lower than the T_m of DNA being used, fibres will form, possibly due to the immediate and faster neutralization of DNA by the PAMAM dendrimers.
- cooling programme: crosslinking *via* DNA hybridization and higher levels of organization are only possible within the DNA/dendrimer-based films when a slow cooling process is implemented, giving the system time to self-assemble.
- ionic composition of the solution: ionic strength and ion composition were shown to be important for film formation. These factors play an important role in DNA strand stability, the interactions between the DNA and PAMAM dendrimers and their surrounding charge network, thereby affecting the interaction and complexation of the different components. Films were only obtained in magnesium-free PBS solution.
- initial pH of the DNA solution and N/P ratio: pH is a key parameter for DNA condensation as it affects the pH-dependent protonation degree of the PAMAM dendrimer. Higher initial pH values prevent DNA condensation due to PAMAM deprotonation, while lower pH values lead to increased interactions between the dendrimer and DNA thereby preventing the posterior hybridization of free

ssDNA strands. On the other hand, charge ratio was shown to be important, with films forming at the same N/P ratio irrespective of the PAMAM dendrimer generation used.

- Dendrimer generation: Higher PAMAM dendrimer generations (G3 or above) were successfully used to prepare the films as their charge density (primary amines) and size allow for interactions with several ssDNA strands. Also, their more spherical shape results in different interactions/arrangements with the DNA. In contrast, lower PAMAM dendrimer generations cannot interact with several ssDNA strands and as such more dendrimers are needed to condense the same amount of DNA. Moreover, their more planar shape may result in different DNA/dendrimer arrangements.

- DNA concentration, size and shape: DNA condensation is a cooperative process. As such, when using low DNA concentrations, more dendrimers and thus higher N/P ratios were required to obtain the films. Here, free dendrimers were more prone to join pre-formed complexes relative to free DNA chains. The use of smaller DNA strands also did not result in film formation as they were completely condensed by the dendrimers and their short length prevented a high degree of crosslinking. Finally, films were also not obtained when using circular pDNA. Here, complete DNA denaturation may be hindered due to structure-related torsion and tension forces.

- Other polymers: Branched, high molecular weight and amine-containing PEI was successfully used to prepare the films. The physical, chemical and structural properties of this polymer are the most similar to that of the G5 PAMAM dendrimer.

Importantly, these films may be scaled up. Films with a larger surface area or increased thickness may be prepared by increasing the flask size or the solution volume, respectively.

Posterior results acquired during the characterization process revealed the following:

- DNA was successfully and efficiently incorporated into the film with approximately 98% of the initial amount of DNA used being incorporated. The presence of the PAMAM dendrimer in the films was also verified when using fluorescent-labelled dendrimers during film preparation.

- Films were stable under acidic conditions. When exposed to an alkaline environment, however, the films immediately dissolved due to PAMAM deprotonation.

- SEM revealed that films were composed of a flat compact bottom layer and a granular interconnected top layer, while zeta-potential measurements showed a positive surface charge attributable to the protonated PAMAM dendrimers at the surface.

- Since the films were revealed to be chiral by circular dichroism, we believe that they are partially internally ordered. Both the slow cooling process and the structure/arrangement achieved by the DNA/PAMAM complexes are likely to lead to this internal organization.

- Films were shown to be very stable for long periods of time under physiological conditions.

- Although films did not allow for cell adhesion, possibly due to the positively charged surface-related cytotoxicity, cells were able to grow in their presence.

- DNA intercalating (*i.e.* DOX) and binding (*i.e.* cisPt) anticancer drugs were effectively loaded into the films, with only DOX undergoing a sustained release over time and consequently cytotoxic activity.

These materials, due to their unique properties, deserve further study using more sophisticated characterization techniques including TEM, rheometry, ellipsometry, etc. Transmission electron cryo-microscopy (cryo-TEM), for example, would reveal details of the organization of the two layers constituting the films and indicate whether similarities or differences in their organization exist. Static and dynamic rheological techniques would provide information on the viscoelastic properties of the film under different experimental conditions, while ellipsometry would indicate physical properties such as thickness, optical constants, crystallinity, refractive indexes, surface/interface roughness and porosity of the film.

Taking the above into account, the possibility of using DNA/polymer-based films in biomedical and other applications should be considered. The possibility of using other amine-containing polymers derived from either natural or synthetic sources may confer desirable properties such as stability, biocompatibility, (bio)degradability, stimuli-responsive behaviour, etc, depending on the desired application of the film. Also, posterior crosslinking may lead to higher stability and simultaneously avoid film dissolution upon exposure to an alkaline environment. This could be an advantage, for example, for alkaline drug loading as a co-adjuvant for anticancer treatment.

Other applications may be envisaged for these materials, such as the use of these films as platforms for DNA transcription. In this case, the DNA containing gene(s) of interest may first be used during film preparation. Cell lysates, for example, would then be used to translate and transcribe the gene(s) into the desired protein(s). Although circular pDNA did not allow for successful film formation, another approach would be to use the same pDNA after linearization. Knowing the gene construct in the pDNA, and if film preparation was successful, this could constitute a good platform for cell-free protein expression in the presence of the right machinery, as well as substrate-mediated gene delivery.

The study of these materials may help to understand other analogous systems, including biological ones, such as the packaging of DNA in the cell nucleus. Exploring supramolecular chirality in the films (or in the fibres prepared when mixing PAMAM with DNA below its T_m) may be used in chirality sensing. Also, supramolecular chirality has been reported to influence cell adhesion and differentiation.

The presence of DNA in the films and its staining with EtBr demonstrates the potential application of this material, for example, in environmental waste detection and in the removal of

DNA intercalators/endocrine disruptors. Also, the affinity of DNA towards noble metals maybe used to synthesize *in situ* nanoparticles with catalytic properties for catalytic reactor applications.

As proof-of-concept studies, the films were tested as cell growth substrates and as platforms for anticancer drug delivery. Although cells did not adhere to the granular top layer of the films, cell adhesion was shown to be improved following surface functionalisation of the films with RGD peptides which are known to mediate cell adhesion. Further studies may be performed to assess the differences in cell adhesion between the granular top layer of the films and the flat compact bottom layer. The fact that cellular adhesion was not observed on the top layer may be a major advantage for biomedical devices where cell/protein adhesion is undesirable (e.g., in stents). The films were also shown to be feasible systems for loading and successfully delivering the DNA-intercalating anticancer drug, DOX. Experiments also showed that DNA non-interacting drugs could not be loaded into the films, which may constitute a limitation. Nevertheless, the DOX-loaded films maybe biomedically applied as a topical plaster for the local treatment of skin cancer. Alternatively, and although an uncommon approach in the clinical setting, the DOX-loaded films may be intratumorally injected into solid tumors. In this case, the acidic extracellular environment around the cancer cells would not affect film stability and would promote the controlled sustained release of DOX from the interior of the mass thereby avoiding huge systemic side effects.

Considering all the above, we may conclude that this new material has to be further investigated and presents numerous potential applications in the technological or in the biomedical fields.

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