UNIVERSIDADE da MADEIRA

Nº de ordem:06/D/2009

TESE DE DOUTORAMENTO

Apresentada à

UNIVERSIDADE DA MADEIRA

Para obtenção do grau de Doutor

José Luís da Silva Santos

FUNCTIONALIZATION OF DENDRIMERS FOR IMPROVED GENE DELIVERY TO MESENCHYMAL STEM CELLS

Júri:

Reitor da Universidade da Madeira	
Doutor Abhay Pandit	Universidade NUI Galway, Irlanda
Doutora Ana Paula Gomes Moreira Pêgo	Universidade do Porto, Portugal
Doutor David Kelham Smith	Universidade de York, Reino Unido
Doutora Helena Maria Pires Gaspar Tomás	Universidade da Madeira, Portugal
Doutor João Manuel Cunha Rodrigues	Universidade da Madeira, Portugal
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José Luís da Silva Santos

Tese submetida à Universidade da Madeira
com vista à obtenção do grau de Doutor em Química na Especialidade de Química de Materiais

Trabalho efectuado sob a Orientação de:
Professora Doutora Helena Maria Pires Gaspar Tomás
Doutor Pedro Lopes Granja

Funchal - Portugal

To my Parents, my Brothers, my Sister and Rosa

ACKNOWLEDGEMENTS

This thesis was only possible to accomplish due to the support of several persons and institutions. To them, I would like to express a deep sense of gratitude.

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I owe a special word of thanks to Dr. Ana Paula Pêgo, who has provided me numerous valuable advices and helpful discussions. She was always nearby to meet and talk about my work, to proofread abstracts and papers and improve their contents.

I am very grateful to all members of the Molecular Materials Research Group who have directly or indirectly contributed for this thesis. In particular, I would like to thank Prof. João Rodrigues that introduced the field of dendrimers as a topic of research in Centro de Química da Madeira (CQM) and with whom I have learnt the beauty of synthetic chemistry. A special thanks to my dear friends Deepti Pandita (thanks for teaching me the importance of viewing all situations with a *carpe diem* attitude), João Figueira and Manuel Jardim (many thanks for the help in NMR experiments), Susana Sargo, Hewerson Tavares, Rita Castro and Elena Oramas for their priceless support, enthusiasm, motivation and for sharing so many good moments.

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This thesis will be incomplete without a special thank to the institutions or entities and projects that provided the laboratory facilities and the financial support to conduct the research. To the Fundação para a Ciência e Tecnologia (FCT) for the financial support through the Ph.D. Grant (Ref. SFRH/BD/19450/2004, co-financed by the programs POCI 2010 and FEDER) and the Project DENDRALGENE (Ref. PTDC/SAU-BEB/71161/2006). To the Portuguese National Nuclear Magnetic Resonance Network (PTNMR-REDE/1517/RMN/2005-POCI2010/FEDER).

To the University of Madeira, CQM (UMa) and INEB/IBMC (University of Porto) for the laboratory and other physical resources that allowed the execution of this work. The financial support of UMa through the “research funds program” is also acknowledged (less is better than nothing!).

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Tatiana Segura (UCLA, USA), respectively, for kindly providing the hBMP-2 and the Luciferase/Green Fluorescent Protein plasmid DNA.

Lastly, I would like to express my gratitude to the most important persons in my life, to whom this thesis is also dedicated.

I express my true and special thanks to my father and my mother for the endless support, unconditional love, concern and enthusiasm.
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I am forever indebted to my ultimate inspiration and unending source of love, Rosa.
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ABSTRACT

Disease, injury, and age problems compromise human quality of life and continuously motivate the search for new and more efficacious therapeutic approaches. The field of Tissue Regeneration and Engineering has greatly evolved over the last years, mainly due to the combination of the important advances verified in Biomaterials Science and Engineering with those of Cell and Molecular Biology. In particular, a new and promising area arose – Nanomedicine – that takes advantage of the extremely small size and especial chemical and physical properties of Nanomaterials, offering powerful tools for health improvement. Research on Stem Cells, the self-renewing progenitors of body tissues, is also challenging to the medical and scientific communities, being expectable the appearance of new and exciting stem cell-based therapies in the next years.

The control of cell behavior (namely, of cell proliferation and differentiation) is of key importance in devising strategies for Tissue Regeneration and Engineering. Cytokines, growth factors, transcription factors and other signaling molecules, most of them proteins, have been identified and found to regulate and support tissue development and regeneration. However, the application of these molecules in long-term regenerative processes requires their continuous presence at high concentrations as they usually present short half-lives at physiological conditions and may be rapidly cleared from the body. Alternatively, genes encoding such proteins can be introduced inside cells and be expressed using cell's machinery, allowing an extended and more sustained production of the protein of interest (gene therapy). Genetic engineering of stem cells is particularly attractive because of their self-renewal capability and differentiation potential. For Tissue Regeneration and Engineering purposes, the patient's own stem cells can be genetically engineered *in vitro* and, after, introduced in the body (with or without a scaffold) where they will not only modulate the behavior of native cells (stem cell-mediated gene therapy), but also directly participate in tissue repair.

Cells can be genetically engineered using viral and non-viral systems. Viruses, as a result of millions of years of evolution, are very effective for the delivery of genes in several types of cells, including cells from primary sources. However, the risks associated with their use (like infection and immunogenic reactions) are driving the search for non-viral systems that will efficiently deliver genetic material into cells. Among them, chemical methods that are promising

and being investigated use cationic molecules as carriers for DNA. In this case, gene delivery and gene expression level remain relatively low when primary cells are used.

The main goal of this thesis was to develop and assess the *in vitro* potential of polyamidoamine (PAMAM) dendrimers based carriers to deliver genes to mesenchymal stem cells (MSCs). PAMAM dendrimers are monodispersive, hyperbranched and nanospherical molecules presenting unique characteristics that make them very attractive vehicles for both drug and gene delivery. Although they have been explored for gene delivery in a wide range of cell lines, the interaction and the usefulness of these molecules in the delivery of genes to MSCs remains a field to be explored. Adult MSCs were chosen for the studies due to their potential biomedical applications (they are considered multipotent cells) and because they present several advantages over embryonic stem cells, such as easy accessibility and the inexistence of ethical restrictions to their use.

This thesis is divided in 5 interconnected chapters.

Chapter I provides an overview of the current literature concerning the various non-viral systems investigated for gene delivery in MSCs. Attention is devoted to physical methods, as well as to chemical methods that make use of polymers (natural and synthetic), liposomes, and inorganic nanoparticles as gene delivery vectors. Also, it summarizes the current applications of genetically engineered mesenchymal stem cells using non-viral systems in regenerative medicine, with special focus on bone tissue regeneration.

In Chapter II, the potential of native PAMAM dendrimers with amine termini to transfect MSCs is evaluated. The level of transfection achieved with the dendrimers is, in a first step, studied using a plasmid DNA (pDNA) encoding for the *β*-galactosidase reporter gene. The effect of dendrimer's generation, cell passage number, and N:P ratio (where N= number of primary amines in the dendrimer; P= number of phosphate groups in the pDNA backbone) on the level of transfection is evaluated, being the values always very low. In a second step, a pDNA encoding for bone morphogenetic protein-2, a protein that is known for its role in MSCs proliferation and differentiation, is used. The BMP-2 content produced by transfected cells is evaluated by an ELISA assay and its effect on the osteogenic markers is analyzed through several classical

assays including alkaline phosphatase activity (an early marker of osteogenesis), osteocalcin production, calcium deposition and mineralized nodules formation (late osteogenesis markers). Results show that a low transfection level is enough to induce *in vitro* osteogenic differentiation in MSCs.

Next, from Chapter III to Chapter V, studies are shown where several strategies are adopted to change the interaction of PAMAM dendrimers with MSCs cell membrane and, as a consequence, to enhance the levels of gene delivery. In Chapter III, generations 5 and 6 of PAMAM dendrimers are surface functionalized with arginine-glycine-aspartic acid (RGD) containing peptides – experiments with dendrimers conjugated to 4, 8 and 16 RGD units were performed. The underlying concept is that by including the RGD integrin-binding motif in the design of the vectors and by forming RGD clusters, the level of transfection will increase as MSCs highly express integrins at their surface. Results show that cellular uptake of functionalized dendrimers and gene expression is enhanced in comparison with the native dendrimers. Furthermore, gene expression is dependent on both the electrostatic interaction established between the dendrimer moiety and the cell surface and the nanocluster RGD density.

In Chapter IV, a new family of gene delivery vectors is synthesized consisting of a PAMAM dendrimer (generation 5) core randomly linked at the periphery to alkyl hydrophobic chains that vary in length and number. Herein, the idea is to take advantage of both the cationic nature of the dendrimer and the capacity of lipids to interact with biological membranes. These new vectors show a remarkable capacity for internalizing pDNA, being this effect positively correlated with the $–CH_2–$ content present in the hydrophobic corona. Gene expression is also greatly enhanced using the new vectors but, in this case, the higher efficiency is shown by the vectors containing the smallest hydrophobic chains.

Finally, chapter V reports the synthesis, characterization and evaluation of novel gene delivery vectors based on PAMAM dendrimers (generation 5) conjugated to peptides with high affinity for MSCs membrane binding - for comparison, experiments are also done with a peptide with low affinity binding properties. These systems present low cytotoxicity and transfection efficiencies superior to those of native dendrimers and partially degraded dendrimers (Superfect®, a commercial product). Furthermore, with this biomimetic approach, the process of gene delivery

is shown to be cell surface receptor-mediated. Overall, results show the potential of PAMAM dendrimers to be used, as such or modified, in Tissue Regeneration and Engineering. To our knowledge, this is the first time that PAMAM dendrimers are studied as gene delivery vehicles in this context and using, as target, a cell type with clinical relevancy.

It is shown that the cationic nature of PAMAM dendrimers with amine termini can be synergistically combined with surface engineering approaches, which will ultimately result in suitable interactions with the cytoplasmic membrane and enhanced pDNA cellular entry and gene expression. Nevertheless, the quantity of pDNA detected inside cell nucleus is always very small when compared with the bigger amount reaching cytoplasm (accumulation of pDNA is evident in the perinuclear region), suggesting that the main barrier to transfection is the nuclear membrane. Future work can then be envisaged based on the versatility of these systems as biomedical molecular materials, such as the conjugation of PAMAM dendrimers to molecules able to bind nuclear membrane receptors and to promote nuclear translocation.

RESUMO

As doenças, acidentes e problemas relacionados com a velhice comprometem a qualidade de vida humana, motivando a contínua procura de novas e mais eficientes abordagens terapêuticas. Ao longo dos últimos anos, o domínio da Engenharia e Regeneração de Tecidos conheceu uma enorme evolução, nomeadamente, devido à combinação dos importantes avanços ocorridos na Ciência e Engenharia de Biomateriais com os da Biologia Molecular e Celular. Em particular, surgiu uma nova e promissora área científica – a Nanomedicina – que tira vantagem do tamanho extremamente pequeno e das propriedades químicas e físicas únicas dos nanomateriais, oferecendo ferramentas poderosas para a melhoria da saúde humana. A pesquisa em células estaminais (CE), células com capacidade de se auto-renovar e de dar origem aos vários tecidos do corpo, constitui também um desafio para as comunidades médicas e científicas, sendo expectável que, nos próximos anos, surjam novas e estimulantes terapias baseadas em CE.

O controlo do comportamento celular (nomeadamente, da proliferação e da diferenciação celular) é de extrema importância aquando da concepção de estratégias no campo da Engenharia e Regeneração de Tecidos. Diversas moléculas de sinalização, tais como citocinas, factores de crescimento, factores de transcrição, etc., a maioria proteínas, têm sido identificadas, sendo conhecido o seu papel na regulação e no suporte do desenvolvimento e regeneração tecidulares. Contudo, a aplicação destas moléculas em processos de regeneração prolongados exige a sua presença contínua, em elevadas concentrações, pois possuem tempos de vida muito curtos em condições fisiológicas e podem ser rapidamente suprimidas do corpo. Alternativamente, podem ser introduzidos nas células os genes que codificam essas proteínas, sendo estes expressos fazendo uso da maquinaria celular e permitindo uma produção controlada e mais prolongada da proteína de interesse (terapia génica). A modificação génica de CE revela-se particularmente atractiva devido à capacidade de auto-renovação e potencial de diferenciação das CE. Para fins de Engenharia e Regeneração de Tecidos, podem ser geneticamente modificadas *in vitro* CE do próprio paciente que, posteriormente, são introduzidas no corpo (por injecção directa ou com auxilio de uma matriz) onde irão modular, não só o comportamento da células nativas (terapia genética mediada por células estaminais), mas também participar directamente na reparação/regeneração dos tecidos.

As células podem ser modificadas geneticamente recorrendo a métodos virais ou não-virais. Os vírus, como resultado da sua evolução ao longo de milhões de anos, são extremamente eficientes na entrega de genes em vários tipos de células, incluindo em células de origem primária. Contudo, os riscos associados ao seu uso (tais como infecções e reacções imunológicas) estão a direccionar a investigação para sistemas não-virais capazes de transferir eficientemente o material genético para dentro das células. Entre eles, estão a ser investigados métodos químicos que fazem uso de moléculas cationicas como transportadores de DNA. Neste caso, a transferência e expressão de genes continua a ser relativamente baixa aquando do uso de células de origem primária.

O principal objectivo desta tese foi desenvolver e avaliar o potencial dos dendrímeros à base de poliamidoamina (PAMAM) como veículos para a entrega/transferência de genes, *in vitro*, em células estaminais mesenquimatosas (CEM). Os dendrimeros PAMAM são moléculas com um baixo índice de polidispersão, altamente ramificadas e nano-esféricas, apresentando características únicas que os tornam veículos atractivos para a libertação de drogas e genes. Apesar de terem sido investigados, ao longo dos últimos anos, como veículos para a entrega de genes numa grande variedade de linhas celulares, a sua interacção e aplicabilidade na entrega de genes em CEM continua a ser um campo por explorar. Neste trabalho, foram utilizadas CEM de origem não-embrionária (células adultas) devido ao seu elevado potencial em aplicações biomédicas (são consideradas multipotentes) e às variadas vantagens que apresentam em relação às células estaminais embrionárias, tais como o seu fácil acesso e a não existência de restrições éticas ao seu uso.

Esta tese encontra-se dividida em 5 capítulos interligados:

O capítulo I fornece uma visão global acerca dos diferentes sistemas não-virais investigados na transferência de genes em CEM. É dada atenção aos métodos físicos, assim como aos métodos químicos que envolvem o uso de materiais poliméricos (naturais e sintéticos), liposomas, e nanopartículas à base de materiais inorgânicos para a entrega de genes. Adicionalmente, faz-se um sumário das actuais aplicações das CEM geneticamente modificadas, usando sistemas não-virais, em medicina regenerativa, com especial ênfase na regeneração de tecido ósseo.

No capítulo II, é avaliada a capacidade dos dendrimeros PAMAM com grupos amino à superfície para transfectar CEM. Em primeiro lugar, foi avaliado o nível de transfecção obtido com estas moléculas usando DNA plasmídico (pDNA) que codifica o gene da *β*-Galactosidase. O efeito da geração do dendrímero, do número de passagens celulares, e da razão N:P (onde N representa o número de aminas primárias no dendrímero e P o número de grupos fosfato na cadeia de DNA) no nível de transfecção é avaliado, tendo-se observado que os níveis de transfecção foram sempre relativamente baixos. Seguidamente, utilizou-se um plasmídeo que codifica a proteína morfogenética do osso 2 (BMP-2), uma proteína conhecida pelo seu importante papel na proliferação e diferenciação das CEM. O teor de BMP-2 produzido pelas células transfectadas foi avaliado através de um ensaio ELISA e o seu efeito nos marcadores osteogénicos foi avaliado através de métodos clássicos que incluíram a actividade da fosfatase alcalina (um marcador precoce da osteogénese), a produção de osteocalcina, a deposição de cálcio e a formação de nódulos de mineralização (marcadores tardios da osteogénese). Os resultados obtidos demonstram que um baixo nível de transfecção é suficiente para induzir a diferenciação osteogénica *in vitro* de células estaminais mesenquimatosas.

Do capítulo III ao capítulo V, mostram-se os resultados obtidos através da adopção de várias estratégias para modificar a interacção dos dendrímeros PAMAM com a membrana celular e, por consequência, aumentar o nível de transferência génica. No capítulo III, as gerações 5 e 6 de dendrímeros PAMAM foram funcionalizadas com o péptido arginina-glicina-ácido aspártico (RGD) - foram feitos estudos com 4, 8 e 16 unidades de RGD por dendrímero. O conceito subjacente a este estudo baseia-se na afinidade dos péptidos RGD pelas integrinas que são receptores celulares expressos em quantidade elevada à superfície das CEM, o que poderá levar, conjuntamente com a formação de "aglomerados" de RGD, ao aumento da transfecção aquando do uso de dendrímeros modificados com péptidos RGD. Os resultados obtidos demonstram que os dendrímeros funcionalizados com péptidos RGD conduzem a níveis de expressão génica mais elevados do que os dendrímeros não-modificados. Adicionalmente, a expressão génica parece depender da interacção electrostática estabelecida entre a parte dendrimérica e a superfície celular, bem como da densidade de RGD nos "nanoaglomerados".

O capítulo IV reporta a síntese de uma nova família de vectores para transferência génica baseada num cerne constituído por um dendrímero PAMAM (geração 5), o qual foi aleatoriamente funcionalizado à superfície com cadeias alquilo hidrofóbicas que variam em comprimento e número. Neste caso, a ideia é tirar partido da natureza catiónica dos dendrímeros e da capacidade de interacção dos lípidos com as membranas biológicas. Estes novos vectores demonstram uma capacidade notável para internalizar pDNA, sendo este efeito positivamente correlacionado com o teor de $-CH_2-$ presente na coroa hidrofóbica. A utilização dos novos vectores também resulta num aumento da transfecção, mas, neste caso, esta é superior para os vectores que apresentam as cadeias hidrofóbicas mais curtas.

Por último, o capítulo V reporta a síntese, caracterização e avaliação de uma nova família de vectores para transferência génica baseado em dendrímeros PAMAM (geração 5) funcionalizados com péptidos que exibem uma elevada afinidade pela membrana das CEM – para efeitos comparativos, todos os estudos foram também efectuados com dendrímeros funcionalizados com péptidos de baixa afinidade para as CEM. Os sistemas sintetizados apresentam baixa citotoxicidade e eficiências de transfecção superiores às do dendrímeros não modificados e, ainda, dos dendrímeros parcialmente degradados (Superfect®, um produto comercial). Ademais, comprova-se que, com esta abordagem biomimética, o processo de transferência génica é mediado pelos receptores à superfície.

Globalmente, os resultados demonstram o potencial dos dendrímeros PAMAM para serem utilizados, com ou sem modificações, em Engenharia e Regeneração de Tecidos. Que seja do nosso conhecimento, esta é a primeira vez que os dendrímeros PAMAM são investigados como veículos de transferência génica neste contexto e usando, como alvo, um tipo celular com relevância clínica. Demonstrou-se que a natureza catiónica dos dendrímeros PAMAM com grupos terminais amino pode ser sinergeticamente articulada com estratégias de funcionalização de superfície, resultando em interacções mais apropriadas com a membrana citoplasmática e no aumento da entrada de pDNA na célula e consequente aumento da expressão génica. Contudo, a quantidade de pDNA detectado dentro do núcleo da célula através de microscopia de fluorescência é sempre muito baixa comparativamente à quantidade existente no citoplasma (há uma evidente acumulação de pDNA na vizinhança do invólucro nuclear), sugerindo que a maior

barreira à transfecção será, provavelmente, a membrana nuclear. A versatilidade dos dendrímeros como materiais moleculares biomédicos poderá ajudar a ultrapassar este problema, nomeadamente através da conjugação, à sua superfície, de moléculas capazes de se ligarem aos receptores da membrana nuclear e, dessa forma, promover a translocação nuclear. Este será, sem dúvida, um tópico interessante de estudo para o futuro.

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SHORT *CURRICULUM VITAE*

José Luis Santos was born in 1982, in Funchal, Portugal. He graduated in Chemistry (4 years course) in the University of Madeira and, in 2005, received the *Best Chemistry Student Award* from the University of Madeira.

In 2005, he was awarded a Ph.D. grant from the *Fundação para a Ciência e Tecnologia* (FCT) and, since then, he is a Ph.D. Student at the University of Madeira, developing his experimental work integrated in the Molecular Materials Research Group at *Centro de Química da Madeira* (CQM), Department of Chemistry, University of Madeira. His Ph.D. work was done under the supervision of Prof. Helena Tomás (CQM and University of Madeira) and co-supervision of Dr. Pedro L. Granja (INEB-*Instituto de Engenharia Biomédica*, University of Porto).

In the period covered by the Ph.D. grant, he participated in 12 Advanced Courses/Summer Schools covering diverse topics related with the theme of his Ph.D. thesis. He 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 his research efforts, he is author or co-author of 6 publications in international peer-reviewed journals (2 published and 4 submitted for publication). His work also resulted in 5 oral presentations and 13 poster presentations mostly in international scientific meetings.

PROFESSIONAL FORMATION

2009- International Symposium "20 years of Biomagical Engineering in Porto" (Porto, Portugal).

2009- 10th Advanced Course in *Cell-Materials Interactions – Self assembly: from nature to clinics* (Porto, Portugal).

2008- 5th Marie Curie Cutting Edge Conference on *Synthesis and applications of self-assembling materials at nano-scale* (Funchal, Portugal).

2007- 8th Advanced Course in *Cell-Materials Interactions – Inflamation in Tissue Repair and Regeneration* (Porto, Portugal).

2007- 1st TERMIS-EU Summer School on *Key Elements of Tissue Engineering* (Funchal, Portugal).

2007- 3rd Joint Summer School on Hybrid Materials, organized in the scope of FAME european research network (Funchal, Portugal).

2007- Gordon Research Conference on *Biomaterials: Biocompatibility / Tissue Engineering* (Plymouth, New Hampshire, USA).

2007- 3rd Marie Curie Cutting Edge Conference on *Biomineralisation of polymeric materials, bioactive biomaterials and biomimetic methodologies* (Funchal, Portugal).

2007- Microscopy course at the Faculty of Pharmacy/University of Porto (Porto, Portugal).

2006- 7th Advanced Course in *Cell-Materials Interactions – Regenerative Medicine* (Porto, Portugal).

2006- 1st Marie Curie Cutting Edge Conference on *New developments on polymers for tissue engineering: replacement and regeneration* (Funchal, Portugal).

2005- 6th Advanced Course in *Cell-Materials Interactions at Molecular level* (Porto, Portugal).

PARTICIPATION IN PROJECTS

- ✓ DENDRALGENE - Design of new gene delivery vectors based on dendrimers, alginate and the RGD sequence for bone tissue engineering. Funding through Fundação para a Ciência e Tecnologia, Ref. PTDC/SAU-BEB/71161/2006 (175000 euros). Researcher of the project since September 2006.
- ✓ DENDRIFAT - Functionalization of dendrimers using fatty acids for improved efficiency as gene delivery vectors. Funding through UMa (458 euros). Researcher of the project during 2008.
- ✓ Novel drug/gene delivery platforms based on the self-assembly of dendrimer-single strand DNA conjugates (Post-Doc project of Deepti Pandita, PhD). Collaborator since 2009.
- ✓ *In Vitro* study of Human Stem Cell behavior on Yb:YAG laser irradiated Titanium in presence or absence of Hydroxyapatite (part of the PhD project of Dr. Hewerson Tavares, visiting researcher in CQM in 2008) . Collaborator during 2008.
- ✓ Cytocompatibility evaluation of bioinspired superhydrophobic poly(L-lactic acid) surfaces (as part of a collaboration with the 3B´s Research Group/University of Minho). Collaborator during 2008.

LIST OF PUBLICATIONS (SCI)

1. Santos JL, Oramas E, Pego AP, Granja PL, Tomás H. Osteogenic differentiation of mesenchymal stem cells using PAMAM dendrimers as gene delivery vectors. *J. Controlled Release* 2009; 134: 141-148 (2008 Impact Factor: 5.690).

2. Alves NM, Shi J, Oramas E, Santos JL, Tomás H, Mano JF. Bioinspired superhydrophobic poly(L-lactic acid) surfaces prevent the adhesion and proliferation of bone marrow derived cells. *J. Biomed. Mater. Res. Part A*. 2009 (in press). DOI: 10.1002/jbm.a.32210 (2008 Impact Factor: 2.706).

3. Santos JL, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Balian G, Tomás H. Receptor-mediated gene delivery using PAMAM dendrimers conjugated with peptides recognized by mesenchymal stem cells (submitted).

4. Santos JL, Oliveira H, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Tomás H. Functionalization of Poly(amidoamine) Dendrimers with hydrophobic chains for improved gene delivery in mesenchymal stem cells (submitted).

5. Santos JL, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Tomás H. Non-viral gene delivery to mesenchymal stem cells: strategies and applications (submitted).

6. Pandita D, Santos JL, Rodrigues J, Pêgo AP, Granja PL, Tomás H. Delivery of the BMP-2 gene into MSCs: a biomimetic approach using RGD nanoclusters based on poly(amidoamine) dendrimers (submitted).

ABSTRACTS IN REFEREED JOURNALS

1. Santos JL, Rodrigues J, Pêgo AP, Granja PL, Tomás H. Novel conjugates of dendrimers and fatty acids for gene delivery applications. *Tissue Eng. A* 2008; 14:S888-S889.

2. Sargo S, Santos JL, Rodrigues J, Pêgo AP, Granja PL, Tomás H. New PAMAM dendrimers/alginate nanoparticles for gene delivery. *Tissue Eng. A* 2008; 14:S886-S886.

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1. Santos JL, Pandita D, Rodrigues J, Granja PL, Pêgo AP, Tomás H. Dendrimers surface engineering for improved cellular uptake and gene delivery to Mesenchymal Stem Cells. 4th International Meeting of the Portuguese Society for Stem Cells and Cellular Therapy - Novel Frontiers in Stem Cell Research. Lisbon, Portugal: April 29-30, 2009.
2. Song W, Veiga DD, Shi J, Oramas E, Santos JL, Tomás H, Alves NM, Mano JF. Bioinspired surfaces with extreme wettability ranges to control cell attachment and protein adsorption. Tissue Engineering & Regenerative Medicine International Society - Asia-Pacific Chapter Meeting 2008 (TERMIS-AP 2008 Meeting). Taipei, Taiwan: November 6-8, 2008.
3. Santos JL, Oramas E, Pêgo AP, Granja PL, Tomás H. *In vitro* osteoblastic differentiation of MSCs using PAMAM Dendrimers as delivery vectors for the hBMP-2 gene. 8th World Biomaterials Congress. Amsterdam, The Netherlands: May 28-June 01, 2008.
4. Santos JL, Oramas E, Pego AP, Granja PL, Tomás H. Osteogenic differentiation of mesenchymal stem cells through gene delivery. 3rd International Meeting of the Portuguese Society for Stem Cells and Cellular Therapy - From Molecular Mechanisms to Therapeutical applications. Faro, Portugal: April 23-24, 2008.
5. Santos JL, Oramas E, Pego AP, Granja PL, Tomás H. *In vitro* osteogenesis via gene delivery. 5th Marie Curie Cutting-Edge Conference - Synthesis and applications of self-assembling materials at nano-scale. Funchal, Portugal: April 14-18, 2008.

POSTER COMMUNICATIONS

1. Santos JL, Pandita D, Rodrigues J, Granja P, Pêgo AP, Balian G, Tomás H. Receptor-Mediated gene delivery in Mesenchymal Stem Cells by PAMAM dendrimers conjugated with Osteotropic peptides. 10th Advanced Course in Cell-Materials Interactions. Porto, Portugal: June 22-25, 2009.

2. Santos JL, Pandita D, Rodrigues J, Granja PL, Pêgo AP, Tomás H. Dendrimers surface engineering for improved cellular uptake and gene delivery to Mesenchymal Stem Cells. 4th International Meeting of the Portuguese Society for Stem Cells and Cellular Therapy - Novel Frontiers in Stem Cell Research. Lisbon, Portugal: April 29-30, 2009.

3. Santos JL, Oramas E, Pego AP, Granja PL, Tomás H. PAMAM dendrimers used as vectors for gene delivery into Mesenchymal Stem Cells. NanoSpain 2008. Braga, Portugal: April 14-18, 2008.

4. Santos JL, Oliveira H, Oramas EG, Pego AP, Granja PL, Tomás H. Gene therapy platform for bone regeneration using dendrimers and osteogenic plasmids systems. Gordon research Conference on Biomaterials: Biocompatibility / Tissue Engineering. Plymouth, New Hampshire, USA: July 22-27, 2007.

5. Santos JL, Oramas EG, Pego AP, Granja PL, Tomás H. Polyamidoamine dendrimer vectors for gene delivery: Physicochemical characterization and transfection efficiency evaluation. 8th Advanced Summer Course in Cell-Materials Interactions. Porto, Portugal: June 18-22, 2007.

6. Santos JL, Oramas EG, Pego AP, Granja PL, Tomás H. *In vitro* gene delivery studies using polyamidoamine dendrimers: physicochemical characterization of dendriplexes and transfection efficiency evaluation. 3rd Marie Curie Cutting Edge Conference - Biomineralisation of polymeric materials, bioactive biomaterials and biomimetic methodologies. Funchal, Portugal: June 4-8, 2007.

7. Santos JL, Granja PL, Tomás H. *In vitro* evaluation of gene delivery in bone marrow stromal cells by polyamidoamine dendrimers. 14th Annual Congress of the European Society of Gene Therapy (ESGT). Athens, Greece: November 9-12, 2006.

8. Rocha-Sousa L, Santos JL, Pelláez-Abellán E, Guastaldi AC, Tomás H. Cytocompatibility modified by spark-anodization, 4º Congresso Latino-Americano de Órgãos Artificiais e Biomateriais. Caxambú, Brasil: August 8-11, 2006.

9. Santos JL, Pêgo AP, Oliveira H, Granja PL, Tomás H. PAMAM dendrimers as gene delivery vectors for bone tissue engineering. 7th Advanced Course in Cell-Material Interactions. Porto, Portugal: June 19–23, 2006.

10. Santos JL, Pêgo AP, Oliveira H, Granja PL, Tomás H. Use of polyamidoamine Dendrimers as gene delivery vectors for bone tissue engineering. 1st Marie Curie Cutting Edge Conference - New developments on polymers for tissue engineering: replacement and regeneration. Funchal, Portugal: June 1–5, 2006..

11. Santos JL, Granja PL, Tomás H. PAMAM dendrimers as vectors for gene delivery into human primary osteoblastic cells – preliminary cytotoxicity studies. XIII Annual Congress of the European Society for Gene Therapy (ESGT). Prague, Czech Republic: October 29 – November 1, 2005.

12. Santos JL, Rodrigues J. Degradation of organic dyes by TiO_2 nanoparticules under visible artificial light or natural solar irradiation. 6th Conference on Inorganic Chemistry (Portuguese Society of Chemistry). Funchal, Portugal: March 31 – April 2, 2005.

13. Santos JL, Rodrigues J. Photocatalytic degradation of Congo Red using WO_3-Preliminary study. XIX Congress of the Portuguese Society of Chemistry. Coimbra, Portugal: April 15-17, 2004.

ORAL COMMUNICATIONS IN CQM AND INEB INTERNAL MEETINGS

1. Santos JL, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Tomás H. Functionalization of poly(amidoamine) dendrimers for improved gene delivery . 4th Materials Line Meeting. Funchal, Portugal: January 30, 2009.

2. Santos JL, Oramas E, Pêgo AP, Granja PL, Tomás H. *In Vitro* osteoblastic differentiation of MSCs using PAMAM dendrimers as delivery vectors for the hBMP-2 gene. 3rd Materials Line Meeting. Funchal, Portugal: January 25, 2008.

3. Sargo S, Santos JL, Rodrigues J, Pêgo AP, Granja PL, Tomás H. PAMAM dendrimers/alginate nanoparticles for gene delivery. 3rd Materials Line Meeting. Funchal, Portugal: January 25, 2008.

4. Santos JL, Granja PL, Tomás H. Gene therapy for bone regeneration. 2nd Materials Line Meeting. Funchal, Portugal: January 26, 2007.

5. Santos JL, Granja PL, Tomás H. Bone Tissue engineering via local gene delivery. 19th Biomaterials Laboratory Meeting. Porto, Portugal: March 23, 2006.

OTHER ACTIVITIES

- ✓ Collaboration in the organization of several scientific conferences:
  - 6th Conference on Inorganic Chemistry (Portuguese Society of Chemistry). Funchal, Portugal: March 31 – April 2, 2005.
  - 1st Marie Curie Cutting Edge Conference - New developments on polymers for tissue engineering: replacement and regeneration. Funchal, Portugal: June 1–5, 2006.
  - 3rd Marie Curie Cutting Edge Conference - Biomineralisation of polymeric materials, bioactive biomaterials and biomimetic methodologies. Funchal, Portugal: June 4-8, 2007.
- ✓ PhD student representative in the scope of the external evaluation of University of Madeira, Portugal, performed by the European University Association (EUA), 2008.
- ✓ Collaboration in several events for enhancing public awareness of science regularly organized by Madeira Chemistry Research Centre (CQM)/Department of Chemistry (University of Madeira):
  - "A Química é Divertida" (Chemistry is Fun) – a project (one week of experimental demonstrations) to promote the scientific culture in Chemistry and Biochemistry among the young people. Collaborator in the period 2004-2008.
  - "Ocupação científica de jovens nas férias" (Scientific occupation of young people during summer holidays) – activity promoted at the national level by "Agência Nacional para a cultura Científica e Tecnológica – Ciência Viva". Collaborator in the period 2004-2009.
  - Participation in a TV program for science popularization among children (Panda Doc series, Panda Channel from Cable TV, 2008).
- ✓ Collaboration in practical lessons (laboratory works) of the disciplines: Biomaterials and Tissue Engineering (Master in Applied Biochemistry, Univ. of Madeira) and Molecular and Cellular Characterization Techniques (3rd year, Degree in Biochemistry, Univ. of Madeira). Collaborator in 2007/2008 and 2008/2009.
- ✓ Collaboration in the supervision of undergraduate biochemistry students during 1 month periods of training at CQM for laboratory skills development. Collaborator in 2008 and 2009.

SECTION 1.

CHAPTER I.

Non-Viral Gene Delivery to Mesenchymal Stem Cells: Strategies and Applications

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Non-Viral Gene Delivery to Mesenchymal Stem Cells: Strategies and Applications*

ABSTRACT

Mesenchymal stem cells (MSCs) can be isolated from several tissues in the body, have the ability to self-renewal, show immune suppressive properties and are multipotent, being able to generate various cell types. At present, due to their intrinsic characteristics, MSCs are considered very promising in the area of tissue engineering and regeneration. In this context, genetic modification can be a powerful tool to control the behavior and fate of these cells and be used in the design of new cellular therapies. Viral systems are very effective in the introduction of exogenous genes inside MSCs. However, the risks associated with their use are leading to an increasing search for non-viral approaches to attain the same purpose even if MSCs have been shown to be more difficult to transfect by this way. Progress has been made in the last years in the development of chemical and physical methods for non-viral gene delivery. Herein, the application of those methods specifically to MSCs is reviewed and their success in the delivery of therapeutic genes discussed. Key issues in non-viral gene delivery to MSCs are addressed and problems that remain to be solved highlighted.

This chapter is based on the following publication:

Santos JL, Pandita D, Rodrigues J, Pêgo AP, Granja PL, Tomás H, Non-Viral Gene Delivery to Mesenchymal Stem Cells: Strategies and Applications (2009), submitted.

1. GENERAL INTRODUCTION

A huge number of studies have demonstrated the importance of mesenchymal stem cells (MSCs) in regenerative medicine and tissue engineering[1-9]. Indeed, they can be isolated from several tissues throughout the body, expanded in culture, and stimulated to differentiate into mesenchymal and non-mesenchymal cell lineages, characteristics that make them attractive in the biomedical area. Furthermore, MSCs are harvested from adult individuals (autologous cells can be used), thus not raising ethical problems in their manipulation and use[10]. Also MSCs have been shown to be able to suppress immune responses in various situations like organ transplantation and autoimmune diseases[9,11]. Indeed, there is now overwhelming evidence that the mechanisms of action of MSCs in tissue repair include not only cell engraftment and differentiation but also signaling through paracrine secretions and cell-to-cell contacts.

MSCs applications can be potentiated using gene delivery approaches[12-18]. Processes such as proliferation, migration, cell-cell and cell-matrix interactions, differentiation, apoptosis, and secretion of soluble signaling molecules can be controlled through the introduction of exogenous genes inside cells that will result in the synthesis of a specific protein (therapeutic protein). In regenerative medicine and tissue engineering, genetic modification is preferred over the exposition of cells to growth factors and cytokines as the short half-life and body clearance of these molecules may imply the use of either high (non physiological) concentrations or repeated administrations to produce the desired biological effect. Also recombinant proteins may not provide the therapeutic benefit of natural proteins[13]. In fact, the power of cell genetic engineering was recently highlighted by experiments that showed the possibility of obtaining pluripotent stem-like cells (induced pluripotent stem cells, iPS cells) by transferring genes encoding for certain transcription factors into non-pluripotent cells[19,20].

A simple strategy of gene therapy can be followed where genes are directly delivered into the site of injury[13,21]. In this case, administration to the target tissue/organ can be done by injection or controlled release from a scaffold. However, using this method, specific gene transfer to MSCs cannot be guaranteed as all cells present in the injury site can be potentially affected, unless some specificity factor is added to the system,. Another, more elegant possibility is to use a cell-mediated gene therapy strategy where MSCs are first genetically modified *in vitro* and, only

after, implanted in the injury site[13,21,22]. During the *in vitro* process, MSCs can be cultured in cell culture dishes or already in a three-dimensional (3D) environment (scaffold) that, after cell colonization, will be implanted in the patient body. In fact, a number of studies have demonstrated that MSCs can be used for cell-mediated gene therapy and as a tool for understanding the molecular mechanisms leading to repair and regeneration of complex tissues and organs[12-18,21,22].

Both simple gene therapy and cell-mediated gene therapy make use of gene delivery systems. An ideal gene delivery system should present various features, like ensure protection of DNA, achieve desired level of gene transfer and expression, exhibit specific targeting, prevent non-specific interactions with blood components, be nontoxic and non-immunogenic, and be cost effective[23-25]. Broadly, gene delivery systems can be divided in two major categories: viral and non-viral systems[14,23-25]. The traditional method to introduce a therapeutic gene into MSCs involves the use of viral vectors, such as lentivirus, retrovirus, adeno-associated virus and adenovirus derived vectors[26-28]. Although viral vectors present high efficiencies in gene transfer (a process called “infection”) and may allow stable gene expression, their clinical applications are currently narrowed due to potential problems, such as oncogenic transformation, pathogenic risk, and induction of immune responses[23-25]. Furthermore, they present difficulty in large-scale production and size limits for exogenous DNA[23-25]. These complications gave rise to substantial efforts in the development of alternative non-viral systems (gene transfer is, in this case, called “transfection”) which may be additionally classified in physical and chemical methods (Figure 1). Physical methods comprise the simplest, but ineffective, way of delivering genes to cells that is to use naked DNA (without any carrier) in contact with cells[14,29]. They also include physical manipulations of the cells that will result in improved gene transfer such as microinjection, particle bombardment, electroporation, sonoporation and laser irradiaton[14,29,30]. Chemical methods imply the use of natural or synthetic materials (the so called “non-viral vectors”) that are able to transfer the genetic material into the cell[31-34]. Non-viral systems offer several advantages, like any or low immunogenicity (thus, more than one application to the patient may be applied), no risk of infectious disease, flexibility towards the molecular size of loaded DNA, and also low cost[23-25]. When non-viral vectors are used, there is also the possibility of engineering them for targeting specific tissues[35-37]. Actually, non-viral vectors offer a great structural and chemical versatility, being possible to manipulate them and to tune their physicochemical properties so that the

barriers for an improved gene delivery and expression will be overcome[31-34]. Unfortunately, non-viral systems present also some drawbacks. On one hand, physical methods are more difficult to be applied in an *in vivo* situation and can cause tissue damage[29,30,38]. On the other hand, for chemical methods: (a) transfection efficiency compared to viral vectors, both *in vitro* and *in vivo*, is low[25,39]; (b) gene expression is transient[25,39]; (c) toxicity may be high[40]; (d) the level of transfection strongly varies with the cell type[14,41,42]. Indeed, most of non-viral vectors have only been tested in cell lines, being difficult to extrapolate the *in vitro* results to a clinical situation. Often, primary cells, as is the case of MSCs, are refractory to transfection using the majority of non-viral vectors[42].

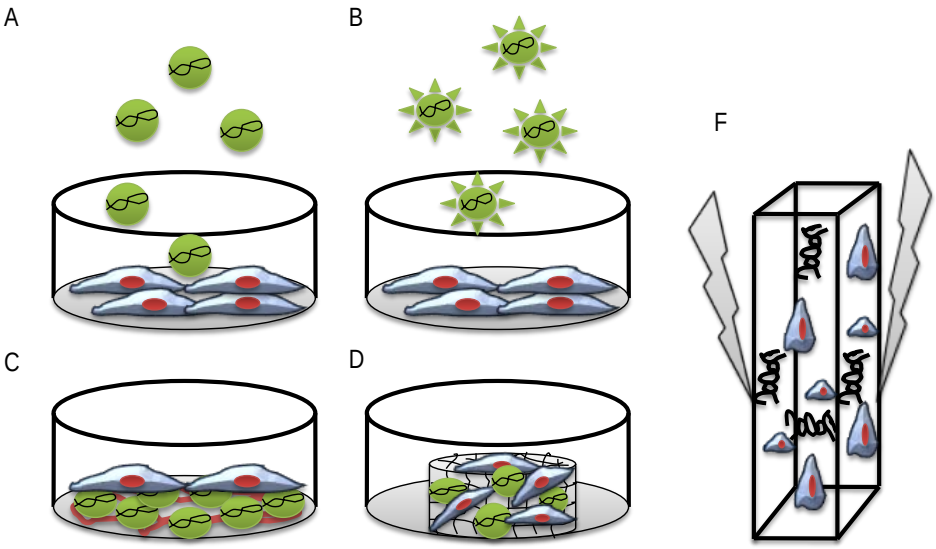

Figure 1. Schematic of DNA delivery to MSCs by chemical (A, B, C and D) and physical methods (F). Non-viral gene delivery vectors are based on a number of different materials that support the packing of DNA into nanometer sized particles. (A) Conventional non-viral gene delivery (bolus delivery). (B) Ligand-receptor-mediated gene delivery. (C) Substrate-mediated gene delivery (reverse transfection). (D) Scaffold-mediated gene delivery (3D environment).

The advantages of exerting genetic control over MSCs behavior and fate associated with the need to move towards non-viral gene delivery represent a current challenge. Research is being done not only focused on the development of better and safer non-viral gene delivery methods but also on the understanding of the mechanisms of the gene delivery process and of its effects on the behavior of MSCs. Herein, we start by brief reviewing the current knowledge on the nature and properties of MSCs, followed by an overview of the use of non-viral gene delivery systems specifically for MSCs transfection. The different strategies used in the delivery of therapeutic genes to those cells for tissue engineering and regeneration applications are

exemplified and discussed. Finally, the main aspects and problems to be solved related with non-viral gene delivery to MSCs are highlighted.

2. MESENCHYMAL STEM CELLS

MSCs were first described in the pioneering studies of Fridenstein, Owen and co-workers in the 60s and 70s[43-48], followed by intensive work on the study of the properties of these cells[49,50]. They were isolated from rat marrow based on their adherence to plastic tissue culture surfaces and were shown to be able to differentiate towards the osteoblastic, adipogenic and chondrogenic lineages. Early studies demonstrated that these cells proliferated rapidly *in vitro* and gave rise to distinct colonies from single precursors, being thus called "fibroblast colony forming units" (CFU-F). The term "mesenchymal stem cells" came after, being first applied by Caplan in 1991, reflecting their "stem cell" nature[51] – that is, capacity for self-renewal (when dividing, they give rise to at least one daughter cell identical to the initial cell) and retention of differentiation potential (MSCs are considered multipotent and can differentiate into specific mesenchymal cell types under the control of environmental cues). Meanwhile, several other names have been attributed to these cells, such as "marrow stromal cells", "mesenchymal stromal cells", "skeletal stem cells", "multipotent mesenchymal stromal cells", and "multipotent adult progenitor cells" This controversy in cell's nomenclature is certainly related with the reduced knowledge that still exists concerning the nature, functions and differentiation potential of MSCs[1,7,52].

For many years MSCs have been isolated from low-density mononuclear cell population of bone marrow based on their selective adherence to plastic surfaces, compared to hematopoietic cells which are non-adherent and can therefore be removed through medium changes[53-56]. MSCs reactivity to monoclonal antibodies was also applied to sort a homogeneous population of cells with defined phenotype by flow cytometry (FACS)[57-59]. Indeed, along the years, several approaches have been investigated for the preparation of a more homogeneous cell population, as is the case of the recently published work of Masoud *et al.* for isolation and culture of MSCs from mouse bone marrow based on the frequent medium change in primary culture and diminishing the trypsinization time[60]. MSCs isolated using this protocol were not contaminated

with hematopoietic cell lineages and readily differentiated into osteoblasts, adipocytes and chondroblasts. However, up to now, all the isolation methods used suffer from lack of absolute specificity due to the lack of specific "markers" that can be employed to select MSCs.

Besides bone marrow, MSCs were also isolated from other adult and foetal tissues – e.g., cord blood, placenta, amniotic fluid, heart, skeletal muscle, adipose tissue, synovial tissue, brain, spleen, liver, kidney, lung, thymus, pancreas, etc.[1-9,61-70]. The wide distribution of these cells in the body raised the hypothesis that MSCs exist in perivascular niches[5]. Furthermore, there are reports about MSCs differentiation not only into mesenchymal tissue lineages but also into non-mesenchymal tissue lineages (such as epithelial, endothelial, and neural cells)[1-9,52,71]. Some discussion exists however around these discoveries and the characterization of these cells and so, in order to distinguish them from MSCs derived from bone marrow (the most studied source of MSCs), it was suggested that they should be called "MSCs-like cells"[9]. In an interesting review, it was even suggested that MSCs under certain conditions (i.e. depending on the cell environment), can de-differentiate returning to a stem state and becoming pluripotent[52].

As explained before, the potential of delivering genes to MSCs (or MSCs-like cells) has been recognized and a broad range of gene delivery systems are now available for the effect. Non-viral systems, being more versatile and safer, are increasingly attractive for the researchers and clinicians that are working in this field.

3. NON-VIRAL GENE DELIVERY TO MESENCHYMAL STEM CELLS

Non-viral gene transfer is normally accomplished using a plasmid DNA (circular molecules of double stranded DNA, pDNA) which contains the gene encoding the protein of interest under the transcriptional control of several eukaryotic regulatory elements and is amplified in bacteria[72]. A number of barriers must be overcome so that the pDNA can efficiently reach the cell nucleus and be transcripted: (a) it must be protected from degradation (and body clearance, in an *in vivo* situation); (b) it must cross the cell membranes (both the cytoplasmic and the nuclear membranes); (c) when chemical methods are used, the complexes formed by pDNA and by the non-viral vectors, and which enter cells through an endocytic pathway, must be released from the

formed endosomes and decomplexation must occur [33,73,74]. The all process of gene transfer will then depend on the preparation, purification and composition of the pDNA, but also on the type of cell used as target, on the non-viral system chosen for the transfection and on the experimental conditions used in the process. Due to all these variables, a strict comparison among the research works published on this subject is difficult to make. However, it is possible to extract general conclusions from a literature survey and to get an idea of the best directions to be followed.

3.1. Chemical Methods

3.1.1. Liposome-based vectors

Cationic lipids have polar heads and non-polar tails, and as such, can self-assemble forming liposomes[33,75]. The positive charge associated with these structures allows their interaction with the anionic molecules of pDNA, being possible to use them as gene delivery vehicles. The DNA molecules are either entrapped in the internal aqueous space of liposomes or bounded on their surface, in both cases forming "lipoplexes". It is believed that, after endocytosis, lipoplexes destabilize the endosomal membrane causing the release of DNA into the cytoplasm. "Lipofection" is a term currently used to mean transfection mediated by liposomes.

Several lipid transfection reagents commercially available, like Lipofectin® and Lipofectamine™, Effectene®, Fugene® 6, and Metafectene®, etc., are now being used for genetic engineering of cells[76-79]. Even if liposome-based DNA delivery systems were one of the earliest strategies used to introduce exogenous genetic material into animal cells, lipofection studies specifically dealing with MSCs are scarce. Hoelters *et al.* transfected human MSCs (hMSCs) with three different liposome-based transfection reagents and a plasmid containing the sequence for enhanced green fluorescent protein (EGFP)[76]. The highest fraction of fluorescent cells was obtained using Lipofectamine™2000 (50%), followed by Metafectene® (22%), and Lipofectamine Plus™ (13%). Often, these commercially available liposome-based vectors are used as reference materials in gene delivery studies for the evaluation of the performance of new gene carriers, as can be noticed throughout this review. In one of these studies, Hamm *et al.* were unable to

transfect hMSCs using 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), Effectene™ and Lipofectamine Plus™ [42]. Indeed, this is not the unique case for which liposome-based vectors were shown to be inefficient for MSCs transfection.

Vanderbyl *et al.* demonstrated the potential utility of mammalian artificial chromosomes (ACEs) for the *ex vivo* gene therapy of MSCs[80]. ACEs are autonomous, being stably maintained in cell nucleus without integrating in cell genome. ACEs delivery was shown to be possible into a variety of mammalian cell lines and primary cells using cationic lipids[81]. Vanderbyl *et al.* work presented the transfer of ACEs into bone marrow derived hMSCs using Lipofectamine Plus™ (4-5 days post-transfection, 11% of the cells were positive for the protein coded in the reporter gene).

In general, the literature reveals that transfection levels attained with liposome-based vectors in MSCs are low and MSCs source-dependent. Also, due to their capacity to interact with biological membranes, the cytotoxicity of these carriers is often considerable and has been pointed out as a limiting factor for liposomes application in gene delivery.

3.1.2. Synthetic polymer-based vectors

Polymers are very versatile molecules that can be tuned to act as gene carriers. Variations in polymer molecular weight, 3-D architecture, size, chemical composition, number of repeating units, degree of branching, side chain length, density, etc., may be used in vector's design. Cationic polymers have been used since last three decades as a material for gene delivery[31-33]. They possess high density of positively charged primary amines which can interact with the negatively charged phosphate groups of DNA to form condensed structures (polyplexes) which enter cells by endocytosis. "Polyfection" is a term currently used to mean transfection mediated by polymers.

Poly-L-lysine (PLL) was the first cationic polymer based gene delivery vehicle[82]. Earlier studies served to elucidate its unnecessary cytotoxicity associated with an intrinsic limited activity and a tendency to aggregate and precipitate depending on the ionic strength of the solution[83,84]. Recently, Farrell *et al.* investigated the potential of PLL as a gene carrier for modification of bone

marrow derived MSCs[85]. *In vitro* studies by flow cytometry showed a poor delivery and transfection efficiency of pDNA to MSCs[85]. In an effort to improve its properties as a gene vehicle, PLL was conjugated with palmitic acid (PA) via amide linkages. PA, a naturally occurring lipid, was shown to improve the interaction of polyplexes with the lipid bilayer of plasma membrane. PLL-PA conjugate resulted in a ~10 fold increase in the cellular uptake which was attributed to better cellular binding and internalization of the conjugate as quantified by flow cytometry. This improved delivery of pDNA was clearly evident in EGFP expression by MSCs[86]. PLL-PA conjugate was further compared with the lipid-based transfection agent Lipofectamine™2000 for plasmid delivery to MSCs. The conjugates achieved a maximum transfection efficiency of ~22%, which was significantly higher than Lipofectamine™2000 mediated transfection (11%). Also increased transfection efficiency with additive effect was observed for PLL-PA and Lipofectamine™2000 combination[87].

Polyethylenimine (PEI) based gene carriers are popular due to their superior gene transfection when compared to other systems, being often used as a reference in gene delivery experiments. PEI exists in both linear and branched forms but the linear molecule is considered more efficient as gene carrier than the branched material, with several linear derivatives of PEI being available commercially as transfection agents, such as ExGen500 and jetPEI™[88-90]. PEI is believed to have a “proton-sponge” effect, that is, it acts as a buffer system inside endosomes leading to pumping of protons and concomitant influx of chloride anions into these organelles, increasing their internal ionic strength and causing their osmotic rupture; lysosomal trafficking of pDNA is then avoided, polyplexes are delivered into the cytoplasm, and pDNA degradation is circumvented. PEI has been studied as a gene carrier over a wide range of molecular weights (0.42-800 kDa) with highest transfection efficiency at molecular weights between 12-70 kDa[91,92]. Recently, several groups have evaluated the transfection potential of PEI into MSCs from different sources. Ahn *et al*. reported transfection up to 10% when pDNA/PEI polyplexes were delivered into rat bone marrow derived MSCs[93]. A good transfection efficiency was achieved at an N:P ratio (N= number of primary amines in the polymer; P= number of phosphate groups in the pDNA backbone) of 16 which was comparable with that obtained with Lipofectamine™. However, at this N:P ratio, only 15% cells were viable as the cationic charge of polyplexes resulted in cytotoxicity. In another study, human adipose tissue-derived MSCs were employed to study the gene transfer properties of pDNA/PEI polyplexes[94]. The highest transfection efficiency

was observed at an N:P ratio of 8, decreasing with further increase in N:P ratio, which was attributed to the increased toxicity. Functionalization of PEI has been used to improve the physicochemical and biological properties of PEI as a gene carrier. A gene delivery system (PEI-RGD) designed for cell targeting was developed by incorporating integrin-binding RGD (arginine-glycine-aspartic acid) peptides in PEI structure[95]. PEI-RGD complexes did not lead to a better transfection and displayed a negative effect on PEI binding to bone marrow derived MSCs. In an effort to improve the transfection efficiency of branched PEI into hMSCs while maintaining cell viability, Saraf *et al.* covalently combined hyaluronic acid (HA) with PEI[96]. They hypothesized that HA could improve the cell targeting as it binds CD44 receptors expressed by hMSCs at their surface and also reduce the toxicity by balancing the cationic amine groups of PEI with the carboxylic groups present in HA. PEI-HA was found to be more cytocompatible and feasible for tissue engineering applications showing cell viability levels of about 86.0 ± 6.7% and a maximum transfection efficiency of 33.6 ± 13.9%. In an interesting study, pDNA/PEI polyplexes were encapsulated in poly(ethylene glycol) (PEG) hydrogels cross-linked with matrix metalloproteinase peptides in an attempt to develop methods for the assessment of gene transfer of infiltrating cells[97]. The infiltrating mouse bone marrow cloned MSCs being transfected multiple times as they encountered new polyplexes during their migration into the scaffold showed gene expression that rose several times through the incubation.

Synthetic polymers can show significant cytotoxicity which limits their clinical application. So, several research works focused on the development of biodegradable non-toxic polymers for gene delivery[32-34]. Such vectors have the advantage to be eliminated after pDNA release, being their degradation products non-toxic. Several biodegradable polymers are being investigated for nucleic acid delivery, such as polyesters, polyurethanes, PLL and PEI based degradable cationic polymers, phosphorus containing polymers, polymers with degradable side chains, polylactic acid (PLA) and poly (lactic-co-glycolic acid) (PLGA)[32-34]. To date, only few studies report the use of biodegradable polymers for transfection of MSCs. Gwak *et al.* developed PLGA nanospheres as vehicles for gene delivery to human cord blood-derived MSCs[98]. The authors were the first to report the low cytotoxicity and long-term transgene expression of PLGA nanospheres compared to PEI. In addition, a library of poly(β-amino esters) end-modified derivatives was recently tested for gene delivery to hMSCs, human adipose-derived stem cells (hADSCs) and human embryonic stem cell-derived cells (hESCds)[99]. The best polymeric vector exhibited transfection efficiencies

of about 27 ± 2% in hMSCs, 24 ± 3% in hADSCs, and 56 ± 11% in hESCds, with high cell viability (87–97%) achieved in all cell types.

3.1.3. Natural polymer-based vectors

Naturally derived non-viral vectors including chitosan, pullulan, gelatin, alginate, dextran and their modified derivatives are being employed for a range of gene delivery applications[31,33]. The use of natural polymers as non-viral vectors is being extensively studied due to their reduced toxicity and because they can provide greater stability in physiological fluids. The biodegradability, biocompatibity and potential of functionalization of these natural polymers can be explored when designing a gene delivery vector. Chitosan, a cationic polysaccharide obtained by deacetylation of chitin, has a strong affinity for DNA and has been investigated as a gene delivery vehicle in several types of cells[100-103]. Variables such as degree of deacetylation, serum concentration and pH of the transfection medium, and stoichiometry of complex were shown to be important in enhancing the transfection efficiency. Although it is the most studied naturally derived non-viral vector, only recently chitosan-DNA complexes have been applied for the transfection of MSCs by Corsi *et al.*[104]. In this study, the transfection efficacy and the cell viability of the complexes on hMSCs, human osteosarcoma and human embryonic kidney cells were compared to Lipofectamine™ 2000. Results showed a transfection efficacy that was cell type dependent and always lower than that obtained using the lipidic vector. However, chitosan-DNA nanoparticles were significantly non-cytotoxic when compared to the complexes formed by Lipofectamine™ 2000 and DNA (for which toxicity was around 50%). When MSCs were employed, transfection efficiency was very low, independently of the chitosan molecular weight tested (150, 400, 600 kDa) showing transfection levels similar to those obtained using naked DNA. A recent study with hMSCs reported higher cell attachment, higher cell viability and desirable gene expression when complexes formed by a plasmid encoding for Bone Morphogenetic Protein-2 (BMP-2) and chitosan nanoparticles were delivered from PLGA/hydroxylapatite (HA) composite scaffolds[105]. Also Hu *et al.* prepared multilayered and gene-functionalized titanium films composed of chitosan and pDNA to investigate the surface-mediated *in situ* differentiation of MSCs[106]. They demonstrated that the films over titanium were beneficial for sustained *in situ* inducing osteoprogenitor cells to differentiate into mature osteoblasts over long time.

Recently, pullulan, a polysaccharide polymer consisting of maltotriose units, was chemically cationized using spermine to prepare a naturally derived non-viral carrier for gene transfection[107]. In this study, a new reverse transfection method was evaluated wherein the spermine-pullulan/pDNA complex was coated onto a culture substrate together with pronectin (cell-adhesion molecule), and then rat MSCs were cultured on the coated substrate. The reverse transfection method significantly enhanced and prolonged gene expression in MSCs when compared with the conventional method. The level and duration of gene expression in MSCs was significantly enhanced when the reverse transfection method was further carried out in a 3-dimensional culture substrate of polyethylene terephthalate (PET) coated with the complex and pronectin and using stirring culture methods. This was attributed to the medium circulation that improved the culture condition of cells in terms of oxygen, nutrition supply and waste excretion.

Hosseinkhani *et al.* used dextran, a polysaccharide, cationized with spermine to complex pDNA[108]. The study was done to enhance the gene transfection of MSCs by combination of a 3-dimensional tissue engineered scaffold and a non-viral gene carrier. A collagen sponge reinforced by incorporation of poly(glycolic acid) (PGA) was selected as scaffold and the polysaccharide derivative-plasmid DNA complexes were there impregnated. The expression levels of MSCs significantly increased when seeded into the scaffolds and cultured in a 3-dimensional environment. In an effort to enhance gene transfection to MSCs and *in vivo* bone formation, the same group also used gelatine, a denatured form of collagen, cationized with spermine to deliver a pDNA encoding for BMP-2[109]. Static and perfusion culture methods were used for these studies to evaluate the effect of the culture system on the transfection efficiency, being the perfusion method superior to the static one – perfusion facilitates the supply of nutrients and oxygen to the cells and the excretion of harmful metabolic products, thus resulting in better cell proliferation and higher gene transfection.

3.1.4. Dendrimer-based vectors

A disadvantage of classical polymers is that they are polydisperse materials (they have undefined molecular weights), being more difficult to assure the reproducibility of their formulations. Dendrimers, which have been classified as polymers although possess well-defined 3D architectures and specific molecular weights, are attractive molecules to be used in gene delivery. They are tree-like molecules, composed of different layers (called “generations”), and a

high multivalent surface. Poly(amidoamine) (PAMAM) dendrimers with amine termini are the most studied dendrimers for gene transfer and have been shown to present an intrinsic ability to associate, condense and efficiently deliver pDNA into a wide variety of cell types[33,74,110,111]. They possess primary amines at the surface and tertiary amines in their interior that, like previously described for PEI, lead to a "proton-sponge" effect. PAMAM dendrimers are also described as possessing low cytotoxicity and immunogenicity[110-112]. Partially degraded PAMAM dendrimers, also called "activated dendrimers", are also available in the market (under the names SuperFect® and PolyFect®) and, like some liposome-based carriers, are also frequently used as reference in gene delivery assays[113,114].

Very recently, our group reported the first study on the use of native PAMAM dendrimers (without any treatment or functionalization) as gene delivery vehicles towards bone marrow derived rat MSCs[115]. The results revealed that the level of transfection was low and dependent on the dendrimer's generation, the N:P ratio and the cell passage number. However, using a plasmid encoding for BMP-2, the low level of transfection was sufficient to induce the *in vitro* osteogenic differentiation of MSCs as shown by several osteogenic markers. Other authors, have found that PolyFect® and SuperFect® were able to deliver pDNA to rat MSCs with transfection efficiencies of 16.29 ± 7.44% and 9.59 ± 3.12%, respectively, both values being lower than that obtained with Lipofectamine™2000 (19.60±3.12)[116]. Studies conducted by Holladay *et al.* presented collagen scaffolds as a reservoir for non-viral gene delivery to rat MSCs[117]. They used complexes composed of SuperFect® and pDNA entrapped in the 3D collagen matrix and results showed a slow and sustained rate of transfection with a longer time of transgene expression. The authors postulated that primary cells, being more sensitive to matrix interactions, would beneficiate from the 3D environment which would act as a powerful adjuvant for gene delivery in these cells.

The great potential of dendrimers in the biomedical area is related with their versatility in terms of chemistry. Dendrimers can be obtained from different building blocks, can have different functional groups, can grow from low until high generations, can retain small molecules in their interior, and can be used as templates for nanoparticle synthesis, etc.[111]. In terms of gene delivery, the functionalization of dendrimers with chemical moieties that have targeting capabilities is being explored as a powerful tool[35,118]. Dendrimeric systems can be tailored to the desired application by linking functional groups at their surface which are recognized by receptors

existent in the cytoplasmic membrane, in the nuclear membrane and/or in the extracellular matrix of the target tissue or cell population. Our most recent work devoted to PAMAM dendrimers consisted in their functionalization with peptides that are recognized by receptors at MSCs membrane[119]. In a first approach, PAMAM dendrimers (generation 5 and 6) were conjugated with up to 16 RGD containing peptides (that bind integrins at cell surface) leading to an improvement of transfection efficiency when compared with native dendrimers. In a second study, PAMAM dendrimers (generation 5) were engineered with osteotropic peptides with high binding affinity for MSCs[120]. This novel system presented low cytotoxicity and transfection efficiencies superior to those of native dendrimers and SuperFect®. By saturating cell receptors with the osteotropic peptide prior to transfection experiments, the transfection efficiency was similar to that of the native dendrimer showing the receptor-mediated nature of the process. In another study, we have functionalized PAMAM dendrimers with alkyl chains of different length at their periphery[121]. These systems showed a remarkable capacity for mediating the internalization of pDNA with minimum cytotoxicity, being this effect positively correlated with the $–CH_2–$ content of the alkyl chains. Gene expression in MSCs was found to be greatly enhanced using these lipidic dendrimers but, in this case, the higher efficiency was shown by the vectors containing the smallest $–CH_2–$ content.

3.1.5. Inorganic nanoparticles

Inorganic nanoparticles have emerged as attractive novel non-viral gene carriers in the last decade[122,123]. Inorganic nanoparticles, such as calcium phosphate, carbon nanotubes, gold, silica, iron oxide, quantum dots and double hydroxide possess several properties suitable for cellular delivery[124]. Although they have been shown to exhibit moderate transfection efficiencies, some of the advantages make them score over other non-viral vectors like availability, low polydispersity, shape control, good storage stability, capability of targeted (by surface functionalization) and controlled delivery, as well as the possibility of having low toxicity.

Until the present, very few studies of DNA delivery to MSCs using inorganic nanoparticles have been reported. Uchimura *et al.* conjugated colloidal gold nanoparticles to DNA/Jet-PEI™ complexes in an attempt to enhance their uptake by hMSCs having in mind their application in cell array-based analyses and in regenerative medicine[125]. Negatively charged gold colloids of

different diameters were used as nano-scaffolds which formed complexes via electrostatic interactions with the positively charged Jet-PEI™ reagent. A 2.5-fold increase in gene transfection was obtained as compared to the control without gold nanoparticles. In an elegant approach, MSCs were coated with acid-functionalized calcium phosphate nanoparticles to prepare an osteoinductive hybrid "living" material[126]. The functionalized HA nanoparticles provided a 3D structural support that stimulated cell differentiation in the absence of osteogenic media. A plasmid encoding for the green fluorescent protein (GFP) was incorporated in the construct and it was shown that gene expression was enhanced when compared to 2D methods involving adherent cells.

3.2. Physical Methods

The non-viral chemical methods discussed till now here are being extensively investigated for improvement of the transfection efficiency, but for their success they need to circumvent numerous barriers to get desired rate and extent of gene delivery. It is postulated that physical approaches may prove to be an efficient tool for transfecting hard-to-transfect cell lines and specially primary cells[29,30]. As for chemical methods, successful *in vitro* and *in vivo* gene expression by physical methods requires efficient DNA delivery while displaying low cell death. To fulfill these requirements, parameters inherent to each method should be properly optimized according to the cell characteristics. Several classical physical methods including electroporation, nucleofection and sonoporation have been used to introduce DNA into MSCs, as well as cutting edge methods that make use of molecular vibrations or nanomaterials as tools for injection of genes into cells.

3.2.1. Electroporation and Nucleofection

In the electroporation technique, a high-intensity electric pulse is applied which makes the cell membrane highly permeable to DNA such that it directly enters into the cytoplasm and is trapped within the cell when the pores close again[29]. In a study, human and rat MSCs (rMSCs) were stably and successfully transfected with pDNA by electroporation at 600V and 100μs[127]. After electroporation, the transient expression of EGFP increased from 12 to 50% with the

exposure to chloroquine that was used to transiently inhibit lysosomes and limit plasmid degradation. However, the presence of chloroquine decreased stable integration. Recently, Ferreira *et al.* optimized the conditions for electrotransfer of the reporter gene lacZ into rat derived MSCs[128]. The pulse electric field intensity, electric pulse type, electropulsation buffer conductivity and electroporation temperature were the parameters studied for optimization of the process. This study confirmed the feasibility of electroporation to introduce genes in MSCs, simultaneously preserving their viability and multipotency. Also, a micro-electroporation concept has been tested with MSCs that employs alternating currents electrical pulses instead of a conventional direct current so as to avoid electrolytic gas bubble formation which could be responsible for the failure of the system[129].

Nucleofection™ is a relative new approach that combines electroporation and cell-type solutions to direct delivery of the DNA into the cell nucleus. This technique has been successfully applied for the transfection of hard-to-transfect cell lines and primary cells, including human bone marrow derived MSCs. Nucleofection™ of hMSCs induced high transient transfection efficiency (73.7 ± 2.9% and 42.5% ± 3.4 of GFP positive cells for U-23 and C-17 pulsing programs, respectively) compared with the non-viral systems FuGENE6 (4.4 ± 2.2%) and DOTAP (6.8 ±4.1%)[130]. U-23 and C-17 pulsing programs were designed for obtaining high transfection efficiency and a high cell survival, respectively. Cell viability was 44.5±3.9% for program U-23 and 94.3±0.9% for program C-17. In a previous study of Hamm *et al.*, the same technology was used for various primary cells, including hMSCs, using ten different pulsing programs[42]. They were able to transfect all primary cells with high efficiencies (45.3±5.6% of GFP fluorescent cells in the case of hMSCs), whereas for the same cells unsatisfactory transfection was obtained with various liposome systems. Cell viability was however low, being 16.5±9.3% for hMSCs. Lakshmipathy *et al.* compared nucleofection and electroporation methods to introduce pDNA into embryonic and MSCs[131]. Nucleofection was found to be superior to the conventional method and transfected both transiently and stably at a rate nearly 10-fold higher. In another study, nucleofection also provided a much better rate of transfer than electroporation particularly in hMSCs[132]. Importantly, the authors showed that transfection efficiency varied with the origin of the cells. Transfection efficiency in rMSCs was about three fold higher than in hMSCs.

3.2.2. Sonoporation

"Sonoporation" is a term employed when mechanical energy in the form of ultrasound is used to transiently enhance the permeability of the cell membrane and increase the efficiency of gene delivery[29]. Although effective, some disadvantages have been pointed out concerning this method as it is limited to a specific tissue type or anatomical region, requires complex equipment and causes damage to the cell membrane[133].

Otani *et al.* used, for the first time, ultrasound in combination with microbubbles (US-MB) to deliver nucleic acids (siRNA) into rMSCs[134]. The transfection efficiency was significantly enhanced but cell viability was significantly affected by US-MB treatment. However, cell viability was dependent on the acoustic intensity and not on the dose of microbubbles.

3.2.3. Molecular vibration

A non-invasive method for high-efficiency DNA transfection based on the principle of electric field-induced molecular vibration was introduced[135]. Genes penetrated into the cytoplasm by eliciting vigorous vibration between molecules and cells. Vibration was induced by two electrodes, which do not have direct contact with the cells nor create a current through the cell suspension during transfection. High efficiency of gene delivery and relatively low cell viability were observed for both human and chick MSCs. The results suggested that the principal cause of low cell viability was the incorporation of exogenous DNA, and not the molecular vibration force.

3.2.4. Nanoinjection

In an effort to develop a low-invasive and direct gene delivery method Han *et al.* injected DNA into single hMSCs using a nanoneedle guided by an atomic force microscopy (AFM) system[136]. Cell viability was maintained without apoptosis signs. Despite of some limitations like availability of AFM apparatus, and time consumption in nanoneedle fabrication and operation, this strategy is unique for accurate single-cell analysis.

Park *et al.* developed a new platform for intracellular delivery of genetic material based on vertically aligned hollow carbon nanotubes arrays of controllable height and showed that that technology efficiently delivered pDNA to hMSCs[137]. They showed that the tubes acted like nanosyringes injecting genes inside cells.

4. THERAPEUTIC APPLICATIONS

There has been a growing body of literature suggesting the inherent potential of MSCs for experimental and clinical applications. MSCs are being explored as cellular vehicles for therapeutic genes, for a wide range of acquired or inherited disorders of bone, cartilage, muscle, tendon and ligaments, and also haemophilia, Parkinson's disease, Alzheimer's disease, and spinal cord injury[1-9,138-146]. In this section, we present an overview of the studies comprising the delivery of therapeutic genes by non viral approaches specifically to MSCs having in mind tissue engineering and regeneration applications.

4.1. Bone tissue

One of the most widely examined applications of non-viral genetically engineered MSCs to date has been in the context of bone tissue engineering and regeneration[2,6]. The use of non-viral systems has been shown to be promising since short-term gene expression is sufficient for bone formation[147]. Several strategies have been proposed for gene therapy applications in bone regenerative medicine (Figure 2). The first one involves the immobilization of gene carriers into nano- or micro-scaffolds designed to control the DNA release and support the cells proliferation at the target site. In this case, the cells are recruited from the body and MSCs are not the only cells to be transfected. Due to the relevance and success of these studies, we decided to include them also in this discussion. The scaffolds can be, or not, associated with non-viral gene delivery vectors that are able to condense and protect pDNA.

In 1990's, one of the pioneer works was devoted to the indirect delivery of bone growth factors *in situ* using gene-activated collagen matrices[148]. A plasmid DNA encoding for bone morphogenetic protein-4 (BMP-4) and a plasmid DNA encoding for a fragment of parathyroid hormone (amino acids 1-34, PHT1-34) were physically entrapped in the collagen sponge to form

a gene-activated matrix (GAM), which was shown to promote wound healing. After migration of surrounding host cells to GAM, cells were able to transiently express BMP-4 and PHT1-34 and to induce new bone formation. GAM with both plasmids worked synergistically in faster bridging of the gap than the delivery of either plasmid alone. In a later study, localized pDNA delivery for approximately 6 weeks was achieved using GAM strategy and a plasmid encoding for PHT1-34[149]. The GAM with highest dose of pDNA (100mg per sponge) showed stable, reproducible, and dose- and time-dependent new bone formation over at least a six month period.

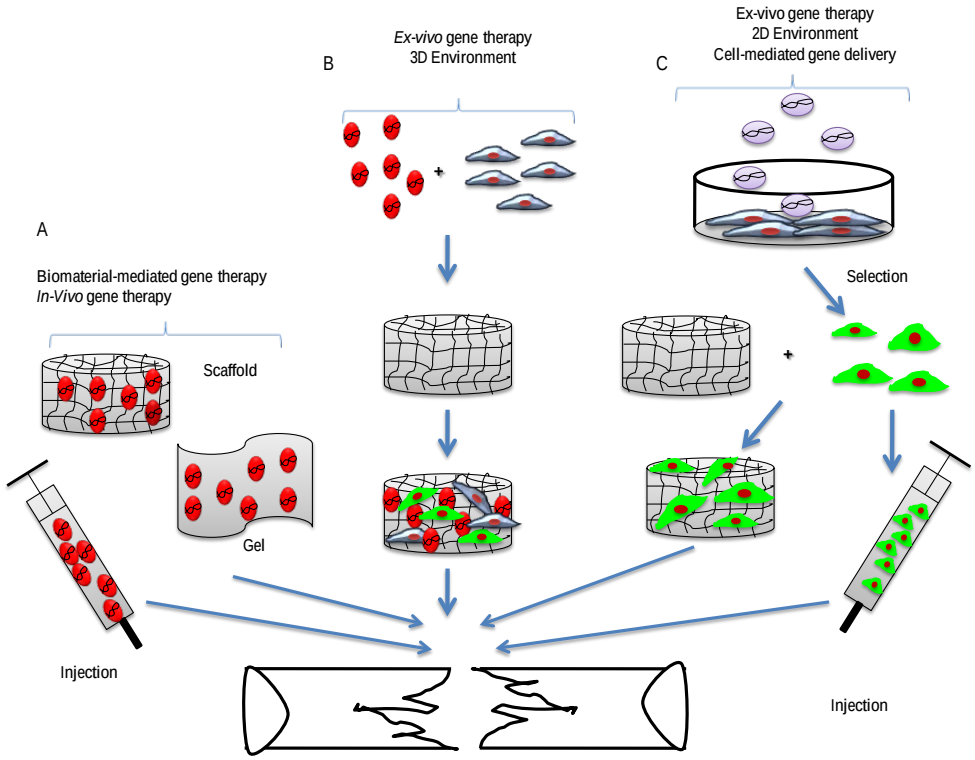

Figure 2. Gene delivery strategies. Therapeutic transgenes may be delivered to bone defects by (A) direct application of gene carrier to the defect or loading the gene carriers onto a biomaterial support (the biomaterial support can deliver the DNA to cells inside the surrounding tissue or can target those cells infiltrating the scaffols or gels), (B) carriers incorporated into the biomaterial support together with cells (hybrid system), or (C) genetic engineering of cells in culture and subsequent implantation into the defect.

In another study, Kasper *et al.* examined and reported lack of enhancement in bone formation using the same strategy with novel hydrogel composites of oligo(poly(ethylene glycol)fumarate) and cationized gelatine microspheres loaded with DNA[150]. It was likely due to the low release of plasmid DNA from the composites attributed to the lack of complete degradation of cationized gelatine microspheres. Kuroda *et al.* modified collagen scaffolds with calcium

phosphate precipitates (CaP) and successfully enhanced the efficiency of plasmid gene transfer *in vivo*[151]. Later, the authors evaluated the performance of the modified GAM using a pDNA encoding for BMP-2 on critical size bone defects[152]. The systems completely bridged the bone defects in less than half the time needed in the previous studies and using a lower DNA dose[148,149]. Thus, the use of pDNA condensing agents in association with GAM may avoid the use of high doses of plasmid which are not practical and might induce some side-effects.

Studies in rat cranial critical-sized defects demonstrated that PLGA scaffolds were able to deliver pDNA encoding for BMP-4 condensed with PEI in a sustained manner and thus to enhance bone formation[153]. Nie *et al.* investigated the bone regeneration capability of BMP-2 plasmid loaded PLGA/HA composite scaffolds in the nude mice[154]. When naked pDNA was used as a coating in fibers surface the release of plasmid was faster and scaffolds performance was better in the first two weeks. At four weeks of treatment, the fibers coated with pDNA/chitosan nanoparticles performed better. The *in vitro* release profiles of pDNA were influenced not only by the DNA presentation, but also by the HA content in the scaffolds. Previously, Ono *et al.* reported the combination of porous HA and cationic liposomes as a carrier for the BMP-2 gene[155]. Conversely, this study reported that osteogenesis was apparently faster in the rats treated with BMP-2 plasmids without scaffolds than in the ones treated with scaffolds. In another study, liposomal vectors carrying pDNA encoding the BMP-2 gene were directly introduced into peri-implant bone defects in pig calvariae with autologous bone grafts resulting in significantly enhanced new bone matrix formation after 4 weeks[78].

The use of physical methods for the *in vivo* gene delivery of therapeutic genes has also been attempted in bone regeneration approaches. In this situation, naked pDNA containing the gene of interest is previously injected into the injured site and, only after, transfection is achieved using physical means. In a study of Kishimoto *et al.*, *in vivo* electroporation was adopted as a gene transfer system for pDNA encoding the BMP-4 gene[156]. More recently, sonoporation based non-viral gene delivery was developed which induced bone formation *in vivo* for skeletal disorders[157]. This direct gene delivery system was simple, cost effective and delivered BMP-9 gene sufficient enough to elicit bone formation in an ectopic site. Though it was not clear which cells were responsible for the formation of bone tissue, the authors speculated that myocytes, muscle progenitors and satellite cells received the gene and expressed it. They refer that the

expressed protein might recruit both bone marrow-derived osteoprogenitor cells in the blood stream and skeletal muscle-derived osteoprogenitor cells within the muscle.

A different and more controllable strategy was used by co-localization of MSCs and genes within a scaffold and by performing transfection in a 3D environment. Also here, non viral vectors can be introduced in the system to protect DNA and facilitate transfection of MSCs. Hosseinkhani *et al.* have published several studies concerned with the *in vitro* transfection of MSCs inside PGA fiber reinforced collagen based scaffolds and using cationized gelatin, cationized dextran or PEI as non viral vectors[108,109,158,159]. Gene expression *in vitro* was positively influenced by the 3D environment, by the use of non-viral condensation agents, and by the perfusion method used in cell culture. Indeed, scaffolds can guide cell organization and spatial arrangement, providing a higher available surface area for cell attachment and proliferation and interfering in the transmission of biochemical and mechanical signals among cells. Furthermore, in a perfusion culture method, medium is recycled and continuously renewed guaranteeing nutrients and oxygen for cells growing in the 3D matrices while wastes and harmful metabolic products from cells can be avoided. The implantation of such kind of systems into the back subcutis of rats resulted in ectopic bone formation[109].

An elegant demonstration comprising a scaffold, a dual factor delivery and MSCs dramatically enhanced bone regeneration[160]. Cells were seeded within PLGA scaffolds containing vascular endothelial growth factor (VEGF) and condensed plasmid DNA (using PEI) encoding for bone morphogenetic protein-4 (BMP-4) and were then implanted into the subcutaneous tissue of mice. All these factors acted synergistically to provide new bone formation of greater quantity and quality in comparison with the single factors alone. Such multi-factor scaffold delivery systems mimic the natural microenvironment more closely and can have broader implications in tissue regeneration and engineering.

MSCs can simply be transfected *in vitro* adherent to the surface of cell culture dishes, in a 2D environment, with or without the aid of non-viral vectors, and then be implanted with or without the support of a scaffold to help bone tissue regeneration. Park *et al*. reported a study where rMSCs were genetically engineered *ex vivo* making use of either adenoviruses or liposomes carrying a pDNA encoding for human BMP-2 and, after, transplanted into rat mandible critical size

defects[79]. Both vectors were shown to be suitable for cell transfection and for bone defect healing achievement. As liposomes have proven sufficient for the purpose and do not present the disadvantages of viral vectors, the authors suggested that they should represent the best vector for future clinical trials of bone regeneration by BMP-2 gene therapy. In contrast, Blum *et al.* showed that among *ex vivo* genetically modified rMSCs by adenoviral, retroviral and cationic lipid vectors, after implantation within titanium fiber mesh scaffolds, the performance of MSCs modified by adenoviral vectors was better in the percent of bone formation in orthotopic critical-size defects created in the rat cranium[161]. Physical methods of gene delivery can also be used for the *ex vivo* genetic engineering of MSCs for posterior implantation. Nucleofection™ based genetic modification of human MSCs was found to be efficient to deliver hBMP-2 and hBMP-9 genes to form bone tissue *in vivo*[162]. Nucleofected MSCs were injected directly in ectopic sites in a mouse model and the harvested transplants showed bone formation 4 weeks post-injection. Using this method, the authors were able to induce moderate levels of BMP-2 expression in an attempt to mimic physiological conditions.

4.2. Cartilage tissue

Cartilage engineering and regeneration may be needed due to trauma or as a result of degenerative diseases. For this, chondrocyte-based therapy may represent a good choice even if it is difficult to obtain sufficient amounts of autologous chondrocytes for clinical application. MSCs are potential candidates as source of cells for chondrocytes as it has been reported that they have the ability to differentiate into chondrocytes by exposure to appropriate chondrogenic differentiation factors[17]. Reviews on gene therapy for cartilage repair have been published but the number of mentioned studies involving MSCs and non viral delivery systems are scarce[17,21].

The design of gene delivery strategies for cartilage regeneration is similar to the one described for bone tissue repair. Scaffolds, non-viral vectors, genes and cells can be combined to attain the desired effect. In addition to genes with anabolic chondrogenic effects, the delivery of genes encoding for anti-inflammatory agents has also been performed as inflammation is often a problem impairing cartilage regeneration[17].

Katayama *et al.* transfected autologous rabbit bone marrow derived MSCs by lipofection (FuGENE®) in a monolayer culture and using the gene coding for cartilage-derived morphogenetic protein 1 (CDMP1)[163]. CDMP1 is a morphogen believed to be involved in skeletal development and joint morphogenesis. It has been shown to modulate MSCs *in vitro* and to maintain cell viability and chondrogenic differentiation *in vivo*. The genetically modified cells were then implanted into full-thickness articular cartilage defects in the rabbit knee joints resulting in an enhancement in cartilage regeneration in comparison with untreated cells. The regeneration occurred with hyaline cartilage filling the defects and reconstruction of subchondral bone over time. Similar articular cartilage repair in rabbits was demonstrated by Guo *et al*.[164]. In this study MSCs were transfected using Lipofectamine™ and a gene coding for transforming growth factor beta 1 (TGF-β_1). TGF-β1 is a multifunctional molecule important in the promotion of cartilage repair, and inhibition of inflammatory and immune responses. Modified MSCs were then seeded in biodegradable poly-L-lysine coated polylactide biomimetic scaffolds *in vitro* and allografted into full-thickness articular cartilage defects of rabbits. Joint repair was improved and occurred with synthesis of hyaline cartilage at the upper portion of the defect, reconstitution of the subchondral bone at the lower portion of the defect and inhibition of inflammatory and immune responses.

In an innovative experiment of Babister *et al*., hMSCs were modified *in vitro* with the gene coding for Sox-9 by Nucleofection™ [165]. Sox-9 is a high-mobility group domain transcription factor believed to have an essential role in chondrogenesis. It is required for the conversion of condensed mesenchymal cells into chondrocytes and prevents conversion of proliferating chondrocytes into hypertrophic chondrocytes[17,166]. The authors developed a “bead-in-bead” system encapsulating transfected (in an alginate “guest” bead) and non-transfected cells (in a chitosan “host” bead) in different localizations of the constructs. *In vivo* studies were performed for a period of 28 days by subcutaneously implantation in mice. The regions with transfected cells showed the presence of chondrocitic cells and a cartilaginous matrix positive for proteoglycans, type II collagen and Sox-9. *In vitro* assays were also done and were in agreement with the *in vivo* data. A previous study of Tsuchiya *et al*. used lipofection to demonstrate the potential of Sox-9 delivery into MSCs for chondrogenesis promotion[167].

4.3. Other tissues

MSCs can differentiate into a myriad of cell types and examples can be found in the literature of genetically modified MSC-based experiments using non-viral systems for the regeneration of tissues other than bone or cartilage. It is the case of cardiac and neural tissues for which this approach seems to be very promising.

It is known that transplantation of MSCs into cardiac tissue induces angiogenesis and improves cardiac functions, thus acting as a tool for tissue repair[168]. MSCs can differentiate into cardiomyocytes and vascular endothelial cells and further act through paracrine pathways to trigger angiogenesis as shown by Miyahara *et al.*[169]. The authors transplanted monolayered MSCs onto scarred myocardium and the engrafted sheet gradually grew to form a thick stratum that included newly formed vessels, undifferentiated cells and few cardiomyocytes. Jo *et al.* further reported that genetically engineered MSCs significantly improved cardiac functions compared to MSCs alone[170]. The authors used a spermine-dextran complex with pDNA encoding for adrenomedullin, a potent vasodilator peptide with anti-apoptotic and angiogenic properties, for genetic engineering of rMSCs. The transfected cells reduced apoptosis *in vitro* and *in vivo* and enhanced the therapeutic efficacy in a rat myocardial infarction model. The proposed strategy represents a promising therapeutic tool to increase the survival of engrafted MSCs and overcome the limitation of a low viability microenvironment like happens in ischemia.

In another study, Potapova *et al.* tested the ability of hMSCs to deliver a biological pacemaker to the heart[171]. hMSCs were transfected with a cardiac pacemaker gene by electroporation and were shown to influence the beating rate *in vitro* when plated onto a localized region of a coverslip and overlaid with neonatal rat ventricular myocytes.

Dezawa *et al.* showed that it was possible to induce cells with neuronal characteristics, without glial differentiation, from MSCs using gene transfection with Notch intracellular domain (NICD, using Lipofectamine™ 2000) and subsequent treatment with molecular factors that induce neuronal differentiation[172]. MSCs expressed markers related to neural stem cells after transfection with NICD, and subsequent trophic factor administration induced neuronal cells. This study showed that MSCs can play a pivotal role as an alternative source of cells for neural

regeneration and highlight the importance of MSCs genetically engineering in the process. The authors further tested the applicability of the induced neuronal cells in the treatment of Parkinson's disease, a neurogenerative disorder, obtaining promising results. Later, Nagane *et al.* used a spermine-pullulan-mediated reverse transfection method to overcome the cytotoxicity issues associated with lipofection in MSCs[173]. Dopaminergic neurons, which are expected to apply to cell-based therapy in Parkinson's disease, were successfully induced from MSCs (derived from monkey, human and mouse) with high efficiency and low cytotoxicity.

5. KEY ISSUES AND PROBLEMS TO BE SOLVED IN NON-VIRAL GENE DELIVERY TO MSCs

From the current knowledge and experience in non-viral gene delivery to MSCs, there are already important general conclusions that can be drawn and that will certainly point out directions for future work. Key issues that must be addressed include:

5.1. Type of non-viral gene delivery system

Chemical and physical non-viral systems have been successfully applied in *in vitro* and *in vivo* situations for MSCs transfection. On one hand, physical methods give rise to higher transfection efficiencies but are less practical and can cause severe damage in cells and surrounding tissues. On the other hand, chemical methods, showing low or moderate levels of transfection, can be less cytotoxic and more versatile. In fact, most of the works show positive effects or therapeutic benefits even with low levels of transfection, revealing that transfection efficiency by itself may not be decisive for the all process. From the literature, it is clear that autocrine and paracrine signalling, as well as cell-to-cell contacts, can result in an amplification of the effects of gene delivery. As an advantage, non-viral delivery vectors can be chemically modified to interact with cell structures, namely with nuclear and cell surface receptors, likely improving gene transfer and toxicity parameters. Indeed, the nuclear membrane has been pointed out as the rate limiting step of the transfection process and the development of a high efficient non-viral vector able to overcome that barrier is still an outstanding challenge. Also, the

search for natural and biodegradable non-viral vectors is evident due to concerns related with cytotoxicity.

5.2. MSCs vs the recruitment of body cells

In vivo gene delivery can be done by simply gene injection or through the controlled release from implanted scaffolds. The use of non-viral vectors to assist the transfection process is advantageous. As exemplified by several works, these strategies are based on the recruitment of neighboring cells or body cells to the injured site and several cellular types can simultaneously suffer transfection. Although good results have been obtained with these approaches, the implantation (either by injection or seeded in a scaffold) of MSCs genetically engineered *ex vivo* – MSCs mediated gene delivery - should constitute a better option as a higher concentration of potential tissue building cells will be present at the injured site, and because such a system will depend on a less number of variables. Moreover the use of MSCs as vehicles for gene delivery may potentiate the expression of therapeutic genes for longer periods whereas the direct administration of genes will present an effect limited in time.

5.3. Source of MSCs

MSCs from different animals and origins within the animal body have been used for non-viral gene delivery. The few reports that made comparisons among the behavior of MSCs of different sources revealed that the extension of cell response may diverge. Further work still needs to be done to understand these differences and to allow a confident extrapolation of the results for the clinical scenario. Moreover, further research on MSCs nature and properties, as well as on the methodologies for MSCs isolation and characterization, is required to optimize genetically modified MSCs-mediated therapies.

5.4. 2D (static culture) vs 3D environment (perfusion culture)

Ex vivo MSCs transfection, with and without the contribution of non-viral vectors, has been performed in 2D monolayer cultures, usually using static cell culture methods, and in 3D environments for which perfusion culture methods are frequently used. The second case implies the previous seeding of MSCs inside a scaffold and has special interest in the tissue engineering area as scaffolds, after implantation, can be important to fill tissue gaps and to guide tissue regeneration. Works done also show that transfection by itself is more efficient in a 3D environment using perfusion culture methods as this approach allows the establishment of cell-cell and cell-substratum interactions that better mimic the *in vivo* situation, as well as the continuous addition of fresh culture media and removal of waste products which will contribute to improve the metabolic state of the cells.

5.5. Genes to be delivered

Non-viral gene delivery does not pose restrictions to pDNA size and type. Literature shows that the choice of genes delivered into MSCs is only dependent on the body tissue to be regenerated, and that genes encoding for a wide range of functional proteins, anti-inflammatory agents, and transcription factors are being used. Although some works studied the effect of the combined delivery of different genes into MSCs, much work needs to be done to understand which genes, in which doses and when should they be delivered to MSCs to obtain the right therapeutic effect.

6. CONCLUSION

From the data accumulated over the last few years, it can be concluded that non-viral systems are actually able to deliver genes into MSCs thus controlling their behavior and fate. The interesting biological characteristics of MSCs together with non-viral gene delivery systems, can offer innovative therapies for disease treatment and tissue regeneration, as shown by the growing number of publications on the subject. In addition, by allowing the control of stem cell signals,

non-viral gene transfer is being used as a tool for understanding the biology and potentialities of MSCs.

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SECTION 2.

CHAPTER II.

Osteogenic Differentiation of Mesenchymal Stem Cells using PAMAM Dendrimers as Gene Delivery Vectors

CHAPTER II.

Osteogenic Differentiation of Mesenchymal Stem Cells using PAMAM Dendrimers as Gene Delivery Vectors*

ABSTRACT

This paper reports the use of different generations of polyamidoamine (PAMAM) dendrimers for the *in vitro* transfection of mesenchymal stem cells (MSCs). A systematic study was carried out on the transfection efficiency achieved by the PAMAM dendrimers using a β-Galactosidase reporter gene system. Transfection results were shown to be dependent upon the generation of dendrimers, the amine to phosphate group ratio and the cell passage number. In all cases, the transfection efficiency was very low. Nevertheless, it was hypothesized that a low transfection level could be sufficient to promote the *in vitro* differentiation of MSCs towards the osteoblastic lineage. To address this possibility, dendrimers carrying the human bone morphogenetic protein-2 (hBMP-2) gene-containing plasmid were used. All quantitative (alkaline phosphatase activity, osteocalcin secretion and calcium deposition) and qualitative (*von Kossa* staining) osteogenic markers were significantly stronger in transfected cells when compared to non-transfected ones. This study not only clearly demonstrates that a low transfection level can be sufficient for inducing *in vitro* differentiation of MSCs to the osteoblast phenotype but also highlights the importance of focusing research on the development of gene delivery vectors in the concrete application.

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1. INTRODUCTION

Stem cell-mediated gene therapy is currently one of the most attractive strategies under investigation for bone regeneration. In this approach, an osteoinductive factor is secreted by the cells that have been genetically modified to express the desired gene[1-7]. Reports on bone regeneration by genetically modified Mesenchymal Stem Cells (MSCs) mainly employ adenovirus or retrovirus vectors carrying bone morphogenetic proteins (BMPs) as therapeutic genes[8-12]. This can be attributed to the high efficiency in gene transfer by the viral vectors, especially in slowly dividing or non-dividing cells. However, the risk of mutagenesis and/or oncogenesis associated with viral systems, as well as their potential immunogenicity, provides a strong motivation for the search of alternative methods. In this scope, several investigations are currently focused on developing high efficiency nonviral vectors, covering a wide range of systems, such as the ones based on cationic polymers, liposomes and calcium phosphates[13-15]. In general, these systems have been shown to be effective in well-established cell lines but have limitations when using primary cell cultures, namely bone marrow-derived MSCs[16,17]. Also, authors often base their conclusions on reporter gene assays and disregard the concrete applications, not exploring the delivery of genes encoding for bioactive agents[18,19].

Dendrimers are spherical macromolecules that consist of a core moiety from which branches radiate. A typical dendrimer consists of a multifunctional central core, branched units (organised in layers called “generations”, G) and surface groups. Polycationic dendrimers entail a particularly interesting system for nuclei acid delivery. These molecules display a well-defined architecture as well as a high multivalent surface moiety (amino termini) to molecular volume ratio[20-22]. Polyamidoamine (PAMAM) dendrimers are amongst the earliest synthesized and commercialized dendritic systems. Since the pioneer work of Haensler and Szoka in 1993[23], these molecules have been tested and used as gene transfer agents in unmodified and modified forms[24,25]. Systems based on the PAMAM dendrimers have been shown to transfect a series of cell types, most of which include cell lines[26]. Gebhart *et al.* compared the performance of several dendrimeric systems and found that the transfection efficiency strongly varied not only with the kind of vector molecule but also with the cell-type[27].

In the present work, the transfection efficiency achieved by different generations of PAMAM dendrimers was evaluated. MSCs were employed here due to their relevance in bone regeneration applications. To our knowledge, this is the first report on the use of polyamidoamine (PAMAM) dendrimers for *in vitro* transfection of MSCs. A β-Galactosidase reporter gene system was used and several experimental conditions were assayed. The transfection efficiency obtained for this system was always very low but it was however hypothesized that such low transfection levels could still be sufficient for the envisaged application, i.e., to improve the in vitro differentiation of MSCs towards the osteoblastic lineage. To confirm this possibility, MSCs were genetically engineered to express human BMP-2 (hBMP-2) using PAMAM dendrimers. The differentiation of the MSCs was studied in the absence of other osteoinductive factors (*e.g.* dexamethasone). This was achieved through the analysis of established markers of the osteoblastic phenotype, including alkaline phosphatase (ALP) activity, osteocalcin, and deposition of a calcified matrix. The results clearly indicate that the PAMAM/hBMP-2 system strongly induces *in vitro* differentiation of MSCs to the osteoblast phenotype.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Generations 5, 6 and 7 of the PAMAM dendrimers, possessing ethylenediamine cores and amine *termini*, were purchased in water solution from Dendritech Inc.. The chemical structures, molecular weights and number of amine surface groups presented by the dendrimers are shown in Table 1. Tissue culture plates were from NUNC. All other materials and reagents were obtained from SIGMA-ALDRICH unless otherwise stated.

2.2. Isolation and culture of bone marrow-derived mesenchymal stem cells

Rat bone marrow-derived MSCs were harvested from the marrow of young adult male (6 weeks) Wistar rats (Charles River Laboratories, Spain) according to a previously described method[28,29]. Briefly, rats were euthanized using 1 ml of 20% pentobarbital. The femurs and tibias

were aseptically excised from the hind limbs. External soft tissue was discarded and the bones were then washed in α-Minimum Essential Medium (α-MEM) containing 5% antibiotic-antimycotic (AbAm) solution. Epiphyses were removed and the bone marrow was flushed out with α-MEM supplemented with 10% Foetal Bovine Serum (FBS, GIBCO), 1% AbAm and 50 $\mu g.ml^{-1}$ ascorbic acid. This was done using a 21-gauge needle syringe. The cell suspension was seeded into six T-75 flasks and cultured at 37ºC and 5% carbon dioxide atmosphere. In order to remove nonadherent hematopoietic cells, the medium was changed on day 4 of culture, and every 3 days thereafter. Between days 7 and 10 the cells were trypsinized using trypsin-EDTA (GIBCO) and subcultured at a density of 2×10^4 $cells.cm^{-2}$ for expansion. When they reached 90% confluency the cells were detached and subsequently used.

Table 1. Molecular weight and number of amine surface groups for the three generations of PAMAM dendrimers used.

Generation	Molecular Weight	Number of Amine Surface Groups
G5	28,826	128
G6	58,048	256
G7	116,493	512

2.3. Culture of human embryonic kidney cells

Human embryonic kidney cells (HEK 293T) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) with high glucose content and GlutaMAX-I containing 10% FBS and 1% AbAm. For transfection experiments, HEK 293T cells were subcultured at a density of 2×10^4 $cells.cm^{-2}$.

2.4. Plasmid propagation and isolation

Plasmid DNA (pCMV SPORT-*β*-Gal, 7.8 kb, Invitrogen) encoding *β*-galactosidase under the control of the cytomegalovirus (CMV) promoter was used as a reporter gene to evaluate the transfection of MSCs by conventional measurement of *β*-galactosidase activity. For BMP-2 gene

delivery, a pcDNA3.1/Hist©/hBMP2 vector/plasmid (8.5 kb) (kindly donated by Prof. Yasuhiko Tabata, University of Kyoto, Japan) encoding a human BMP-2 sequence inserted downstream the CMV promoter was used. The plasmids were amplified in *Escherichia Coli* host strain, DH5α, grown overnight in Luria-Broth Base medium containing ampicillin. Afterwards, the plasmids were isolated and purified using a Qiagen Plasmid Maxi Kit (Qiagen). The isolated plasmids were dissolved in a small amount of ultra-pure water and the concentration of plasmid DNA in solution was determined from the UV absorbance at 260 nm. For evaluation of plasmid purity the absorbance ratio at 260 and 280 nm was measured and was always found to be in the range of 1.7 and 1.9. The plasmid integrity was assessed through agarose gel electrophoresis.

2.5. Cytotoxicity studies

The cytotoxicity of the dendrimers and polyplexes was evaluated by the MTT assay. MSCs were seeded on 96-well plates at a density of 1.5×10^4 cells.cm^{-2} and cultivated in 100 μl of media. After 24 h the medium was removed, the cells were washed once with Phosphate Buffered Saline solution (PBS) and 85 μl of fresh medium containing 5% FBS was added. Then, 15 μl of the dendrimers and polyplexes solutions were added to each well and incubated further for 4 h. The β-galactosidase reporter gene carrying plasmid was used in these cytotoxicity experiments and was kept constant at a concentration of 1 μg.cm^{-2} of plate surface. The polyplexes solutions were prepared by mixing 50 μl of α-MEM containing the adequate concentration of dendrimer with an equal volume of α-MEM containing the adequate concentration of plasmid DNA. The mixture was gently vortexed and incubated for 20 min at room temperature prior addition to the wells. The final concentration of dendrimers in polyplexes solutions, in the wells, ranged from 0 to 1000 nM. As the number of amine groups in the periphery of the dendrimers double in each generation, these conditions correspond to a range of N:P ratios tested of 0-13.3, 0-26.6 and 0-53.2 for the G5, G6 and G7 dendrimers, respectively.

The polyplexes solutions were removed from the wells after 4h and replaced with fresh medium. The cells were incubated for 44 hrs, followed by the addition of 10 μl of MTT stock solution (5 mg.ml^{-1}) per well. After further incubation for 3 h at 37ºC the medium was removed and 100 μl of DMSO was added to dissolve the formazan crystals formed by metabolically active

cells. Absorbance was measured at 550 nm and the cellular metabolic activity was expressed as a percentage of the value obtained for the control (untreated cells).

2.6. Transfection assays using a *β*-galactosidase reporter gene

For transfection assays using a *β*-galactosidase reporter gene carrying plasmid, MSCs were seeded (1.5×10^4 cells.cm^{-2}) into 24-well plates and grown in 1 ml of culture medium for 24 h prior to transfection. At the time of transfection, the medium was replaced by 500 µl α-MEM containing 5% FBS. Polyplexes solutions were prepared as described in the last section for three N:P charge ratios (see Table 2). 50 µl of α-MEM containing the adequate concentration of dendrimer were added to an equal volume of α-MEM containing the adequate concentration of plasmid DNA. The mixtures were gently vortexed and incubated for 20 min at room temperature and then added to the wells. The polyplexes were incubated with the cells for 4 h and then removed and replaced with α-MEM containing 10% FBS and ascorbic acid. After 48 h, transfected and non-transfected cells (control) were washed with PBS, lysed in lysis buffer (0.25 M Tris, pH = 8) and either analyzed immediately or stored at –20ºC for later analysis. The *β*-galactosidase activity was detected spectrophotometrically (405 nm) using *o*-nitrophenol galactoside as the substrate and purified *β*-galactosidase enzyme as the primary reference standard. The *β*-galactosidase activity was normalized to the protein concentration in the lysed cell samples, which was measured by the BCA protein assay method. Each experiment was carried out two times independently.

Cytochemical X-Gal staining was performed to visualize the β-galactosidase expression in transfected cells. The cells were incubated with a glutaraldehyde-formaldehyde fixing solution and then with a solution containing 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal, BDH-PROLABO). In transfected cells, *β*-galactosidase cleaves 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) to produce a blue stain.

2.7. Transfection assays using a plasmid carrying the hBMP-2 gene

For dendrimer-mediated hBMP-2 gene delivery, MSCs (1.5×10^4 cells.cm^{-2}) were plated in 12- or 24-well plates at 37ºC in culture medium for qualitative and quantitative tests, respectively. After 24 h, the cells were transfected as described before, but now using the hBMP-2 plasmid (final dose of 1 μg.cm^{-2}). After 4 h the medium was removed and the cells were washed with PBS. The cells (transfected and non-transfected) were cultured in complete medium containing 10% FBS, 50 μg.ml^{-1} ascorbic acid and 5 mM of β-Glycerophosphate. The medium was replaced every 2 days. At different time points, the cultures were characterized for osteogenesis evaluation.

Table 2. Concentration of dendrimers in solution for the N:P charge ratios used in the transfection experiments (the concentration of DNA was always kept constant at a value of 1μg.cm^{-2} of plate surface).

N:P ratio	Concentration of dendrimers (nM)		
	G5	G6	G7
5:1	376	188	94
8:1	602	301	150
10:1	752	376	188

2.8. Expression of the BMP-2 protein

Three days after transfection, the expression level of BMP-2 gene was measured. 100 μl of the supernatant was collected and the amount of BMP-2 protein was determined by a human BMP-2 ELISA Kit (Human BMP-2 Super X-ELISA, Antigenix America INC., USA) according to the manufacturer's instructions.

2.9. Alkaline phosphatase activity assay

The intracellular alkaline phosphatase activity was quantified by using a colorimetric endpoint assay that involves the hydrolysis of *p*-nitrophenyl phosphate to *p*-nitrophenol. A standard curve was generated by using serial dilutions of *p*-nitrophenol, and the ALP values were normalized to the total protein in cell lysates. Cells were harvested after 7, 14 and 21 days in culture. Each experiment was carried out two times independently.

2.10. Osteocalcin (OC) secretion assay

The amount of OC secreted to the culture medium was measured by using a commercially available sandwich ELISA assay (BT-490), specific for rat osteocalcin (BTI, Stoughton, MA). The ELISA assay is based on two goat OC antibodies, recognizing both the carboxylated (C-terminal protein) and the decarboxylated (N-terminal protein) rat osteocalcin. Media samples were collected at 14, 21 and 28 days of the culture period and stored at -20 ºC for later analysis. These samples were thawed on an ice bath, vortexed for 10 s and analysed according to the manufacture's recommendations. The lowest detectable level of OC with this assay kit is 0.5 $ng.ml^{-1}$. Samples were run in triplicates and compared against rat osteocalcin standards.

2.11. Calcium deposition assay

Deposited calcium on the cell layers was measured by using ortho-cresolphthalein complexon method (Wako, Calcium C test). Briefly, non-transfected and transfected MSCs were cultured during 21 days in complete medium. At this time point, the cells were washed twice with PBS and the calcium content was quantified by dissolving the cell matrix in 0.25 ml of 0.6 N HCl at room temperature for 24 h, with gentle shaking. The calcium content was quantified according to the manufacture's recommendations and the values normalized to the total protein concentration.

2.12. Von Kossa staining

Mineralization was assessed qualitatively by the von Kossa staining. For that purpose, cells were first fixed using the same procedure used for ALP staining. Cells were then incubated with 2.5 wt-% silver nitrate solution under ultra-violet light for 45 min. Then, the plates were rinsed with deionised water followed by treatment with 5% sodium thiosulfate for 3-5 min.

2.13. Statistical analysis

Data were analyzed by two-way analysis of variance, and then differences between means were analyzed using the Fisher's protected least significant difference multiple comparison test. Calculations were performed using SPSS® version 15.0.

3. RESULTS

3.1. Cytotoxicity studies

The toxicity of the PAMAM dendrimers towards MSCs was evaluated using the MTT assay. This was done for the dendrimers and for the polyplexes based on dendrimers and the *β*-galactosidase reporter gene carrying plasmid. The cytotoxicity profiles shown in Figure. 1 indicate the percentage of metabolic activity of the transfected cells in relation to the unexposed, control cells. The studies involved generations 5, 6 and 7 of the PAMAM dendrimers, at concentrations ranging from 0 to 1000 nM. This interval includes dendrimer concentrations used in later transfection assays (table 2). For polyplexes cytotoxicity evaluation, the plasmid DNA concentration was kept constant at 1 $\mu g.cm^{-2}$ of plate surface. This value was selected during preliminary gene transfection experiments as it provided the highest levels of gene expression allowing for cell viability. Larger amounts of plasmid DNA implied the use of higher dendrimer concentrations, and consequently decreased cellular viability. PAMAM dendrimers (G=5-7) displayed concentration- and generation-dependent toxicity. Toxicity was observed to increase with increasing dendrimer concentration, and followed the order of G5 < G6 < G7 (Figure 1A),

confirming that a high density of cationic amines can be damaging to cell membranes. Polyplexes cytotoxicity followed the same trend (Figure. 1B).

Due to the differences in the number of amine surface groups among G5, G6 and G7 dendrimers, the dendrimer concentration used during the transfection experiments varied with the dendrimer generation number for the fixed N:P ratios used (table 2). The highest dendrimer concentrations used corresponded to the N:P ratio of 10 and were 752 nM for G5 dendrimers, 376 nM for G6 dendrimers and 188 nM for G7 dendrimers. In these conditions, the G5 dendrimers, at N:P ratios of 8 and 10, were the more cytotoxic, even if around 70% of metabolic activity percentage values were achieved.

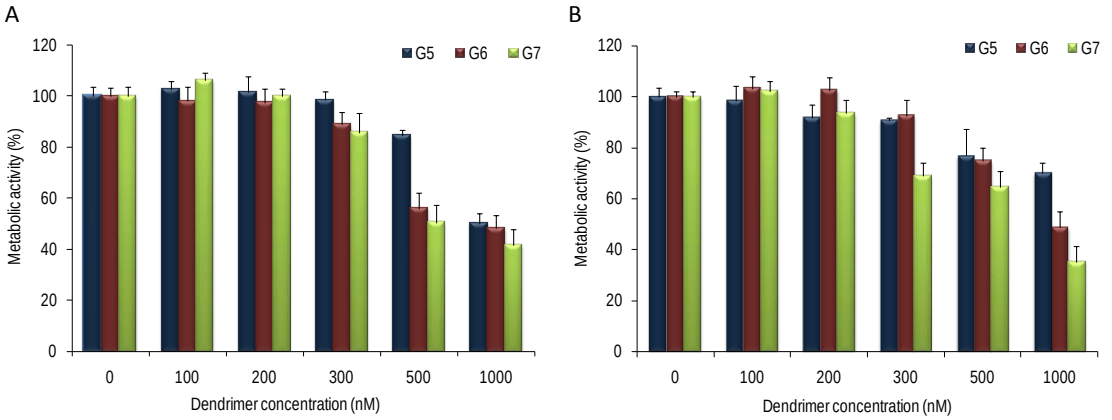

Figure 1. Effect of dendrimer concentration on the percentage of metabolic activity of MSCs in relation to the control (0 nM): (A) for dendrimers and (B) for polyplexes - this range includes dendrimer concentrations used in later transfection assays (table 2). Values represent means from six replicates ± s.e.m..

3.2. Transfection assays using a *β*-galactosidase reporter gene

The ability of the PAMAM dendrimers to transfect MSCs *in vitro* was assayed using generation 5, 6 and 7 dendrimers and a plasmid encoding the *β*-galactosidase reporter gene. Assays were carried out at different N:P ratios (Figure 2A). Two factor ANOVA revealed that the level of gene expression was always significantly higher at the highest N:P ratio used (10:1) for all the dendrimer generations tested ($p<0.05$, power = 1). At this N:P ratio, results obtained for G5 and G6 dendrimers were significantly higher than those obtained for G7 ($p<0.001$, power = 1). Higher transfection efficiencies were then obtained in conditions of higher cytotoxicity.

Transfection with lower generation PAMAM dendrimers (data not shown) or with naked DNA did not result in any significant increase in *β*-galactosidase activity when compared to control cultures. Moreover, the extent of transfection was affected by the passage number of the cells (Figure. 2B). Increasing the cell passage number from 2 to 4 resulted in a marked decrease in gene expression levels in MSCs transfected with either G5 or G6 dendrimers ($p<0.05$, power = 1). Based on these results, MSCs from passage 2 were always used in the transfection experiments involving the hBMP-2 encoding plasmid.

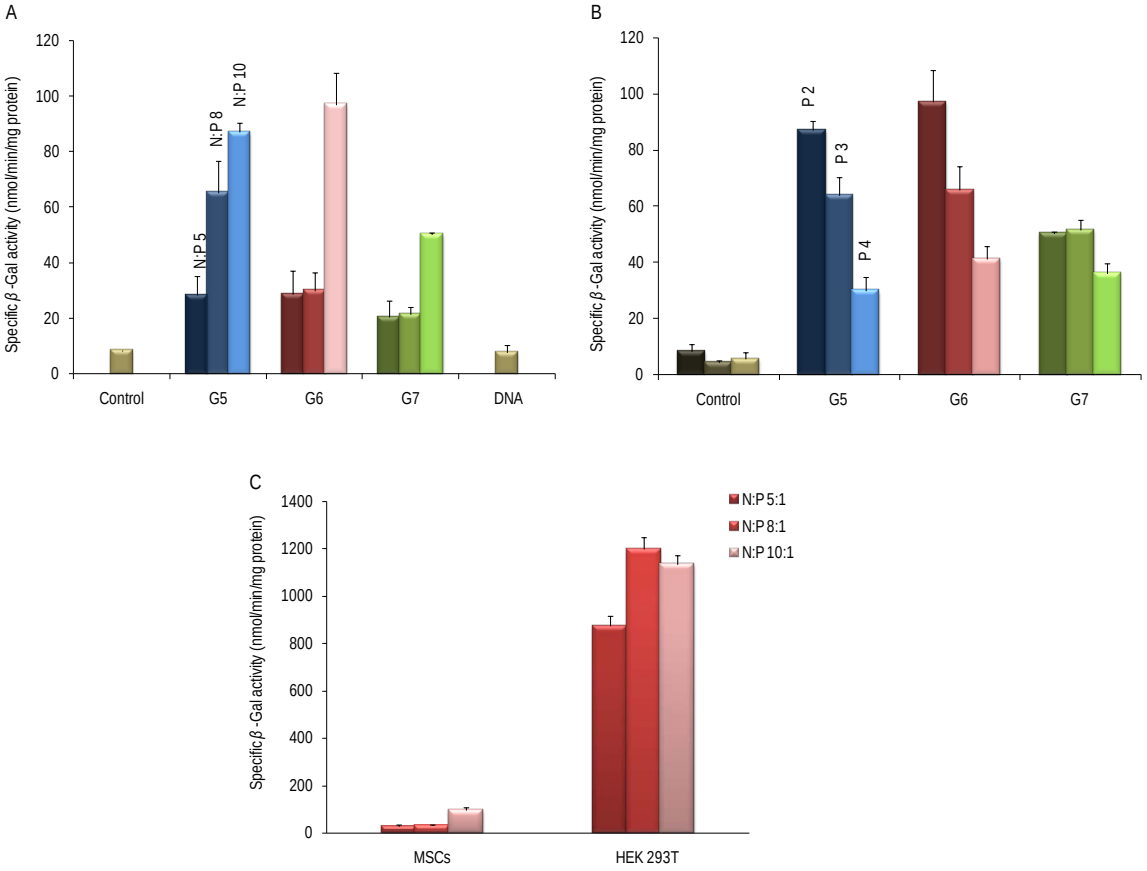

Figure 2. *In vitro* *β*-Galactosidase activity in MSCs 2 days after transfection using G5-7 PAMAM dendrimers at (A) different N:P ratios, using cells from the second passage, and (B) at a fixed N:P ratio of 10, using cells from different passages. For comparison, the *β*-Galactosidase activity in MSCs and HEK 293T cells, 2 days after transfection and using G6 PAMAM dendrimers, is also presented (C). Results for non-transfected cells (control) and naked DNA (DNA) are also shown. Values represent means from 6 replicates ± s.e.m.

Similar transfection experiments were performed using human embryonic kidney 293T cells. The results obtained here clearly indicate that the transfection levels attained using MSCs are very low ($p<0.001$, power = 1). This is exemplified by the quantitative comparison of *β*-

galactosidase activity in both cell types after transfection using generation 6 PAMAM dendrimers at a N:P ratio of 10 and under the same experimental conditions (Figure 2C). A qualitative comparison based on X-Gal staining (Figure. 3), a substrate that turns blue when hydrolyzed by *β*-galactosidase, revealed a high number of blue cells in HEK 293T transfected cultures, and a scarce number of blue cells in MSCs transfected cultures.

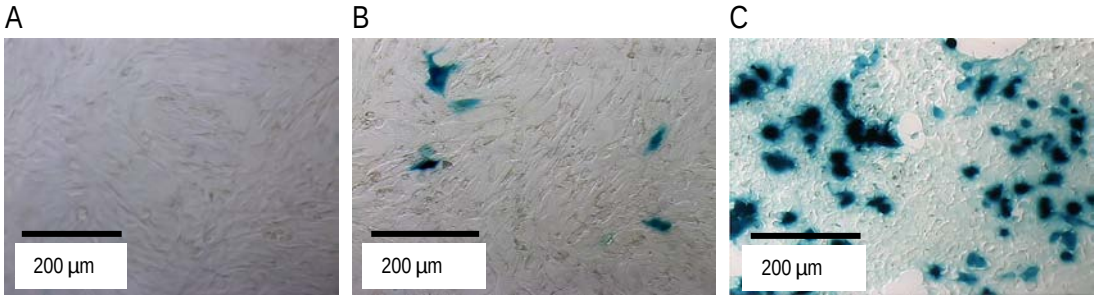

Figure 3. X-Gal Staining for (A) non-transfected MSCs, (B) transfected MSCs and (C) transfected HEK 293T cells, using G6 dendrimers at a N:P ratio of 10.

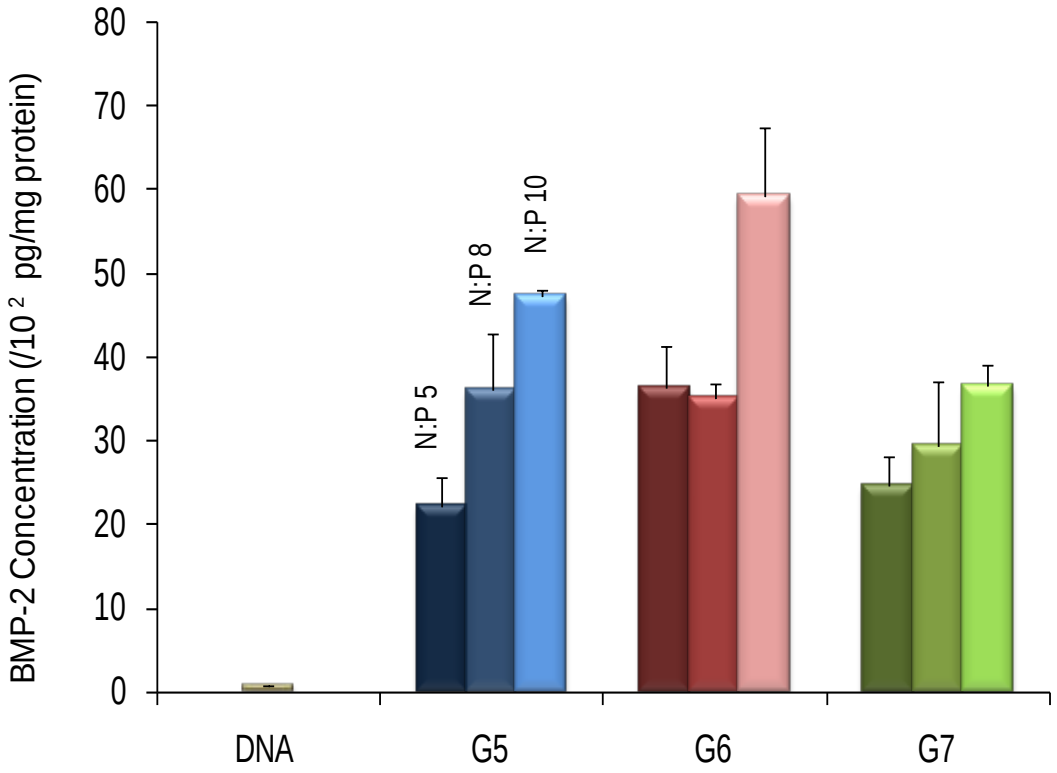

Figure 4. *In vitro* BMP-2 expression by MSCs 3 days after transfection. Values represent means from three replicates ± s.e.m..

3.3. Transfection assays using a plasmid carrying the hBMP-2 gene

The first set of experiments involving the use of the *β*-galactosidase reporter gene revealed very low levels of transfection efficiency. The results of BMP-2 expression 3 days after transfection (Figure 4) showed a pattern similar to the results obtained with the β-galactosidase reporter gene. BMP-2 expression was higher for the N:P ratio of 10. At this ratio, results obtained for G5 and G6 dendrimers were significantly higher than those obtained for G7 ($p<0.5$, power =1).

Anyway we decided to determine whether the low levels of gene expression obtained using the hBMP-2 containing plasmid would still be sufficient for the *in vitro* differentiation of MSCs towards the osteoblastic lineage. In order to assess this, established markers of the osteogenic phenotype were evaluated: alkaline phosphatase (ALP) activity, an early marker of osteogenesis; osteocalcin (OC) secretion, a non-collagenous matrix protein that is only synthesized by mature osteoblasts, and is not present in the early stages of calcification, being expressed only after calcified deposits start to form; and extracellular matrix mineralization, the ultimate step in osteogenic differentiation.

Figure 5 shows the time course of the ALP activity (normalized to the total protein content) presented by the transfected MSCs. Significantly higher levels of ALP activity were always observed for cells harvested at day 7 compared to those harvested on days 14 and 21 ($p<0.05$, power = 0.99). From this it is clear that the expression of ALP by cells is a transient event with a peak at the beginning of the osteogenesis process. At days 7 and 14, the ALP activity values for the transfected MSCs were always significantly higher ($p<0.05$, power = 1) than those obtained for the non-transfected cells. However, the effects of the different dendrimer generations and N:P ratios tested were not distinguishable.

The OC content secreted into the culture media was determined at days 14, 21 and 28, and is presented in Figure 6. A peak in the levels of secreted OC was always observed on day 21, revealing the late nature of this marker of osteogenesis. The results at this time-point clearly highlight that the transfected MSCs secreted significant higher amounts of OC than the non-transfected cells ($p<0.001$, power = 1). In fact, the OC content in culture was observed to increase with an increasing N:P ratio (significant differences were detected for the G6 and G7

dendrimers). As for ALP activity, the effects of the different generations of dendrimers were not distinguishable.

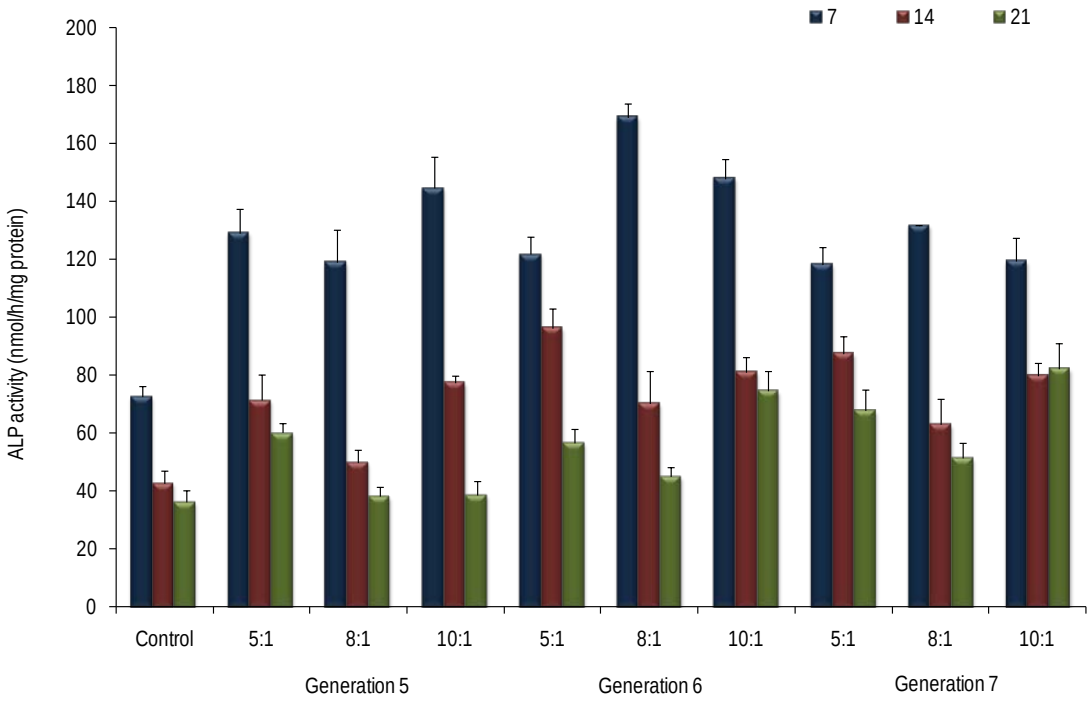

Figure 5. Time course of ALP activity presented by MSCs transfected with a plasmid DNA containing the hBMP-2 gene using generations 5, 6 and 7 of PAMAM dendrimers at various N:P charge ratios. The values represent means from six replicates ± s.e.m..

To further examine cell differentiation in the osteoblastic lineage, the calcium content on the extracellular matrix was assessed at day 21. The calcium deposition assay data (Figure 7) indicated that the transfected cells had accumulated significantly higher levels of calcium when compared with the control group (non-transfected cells-$p<0.001$, power = 1). The highest levels of deposited calcium were observed for the cells transfected with the G5 and G6 dendrimers, using a N:P ratio of 10. For G5, it was clear that an increase in the N:P ratio resulted in a progressive increase in mineralized matrix deposition ($p<0.001$, power = 0.998).

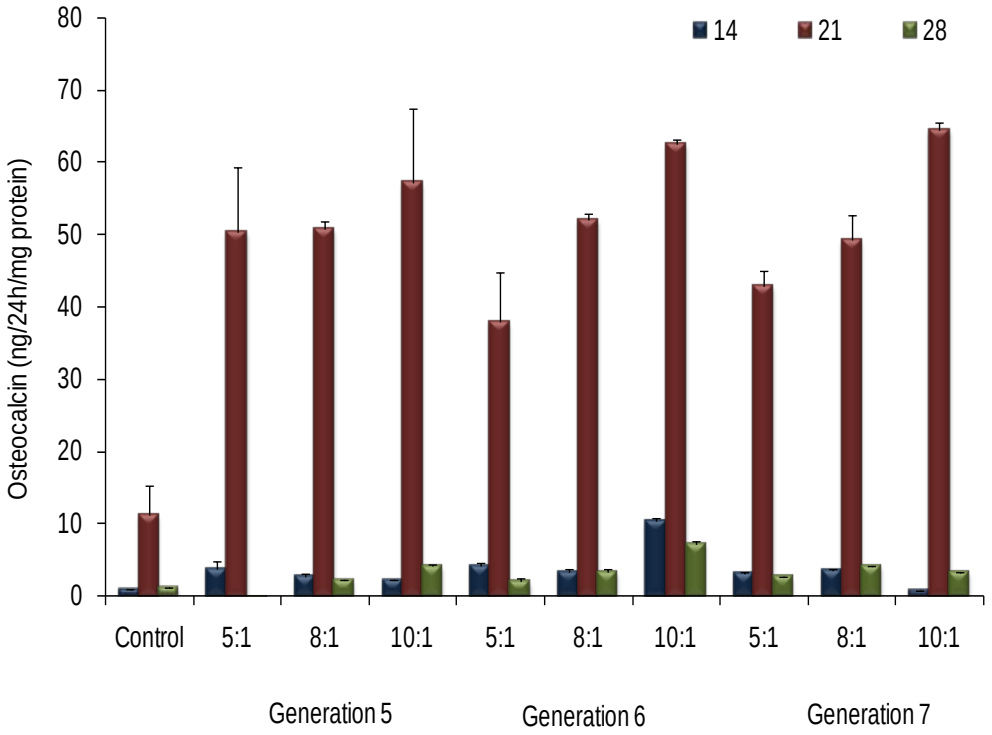

Figure 6. Time course of osteocalcin content secreted by MSCs transfected with a plasmid containing the hBMP-2 gene using generations 5, 6 and 7 of PAMAM dendrimers at various N:P charge ratios.The values represent means from three replicates ± s.e.m..

The presence of a mineralized matrix on day 21 was also confirmed by *von Kossa* staining. The expression of BMP-2 by the transfected cells dramatically enhanced matrix mineralization (Figure 8). This was detectable by the typical black colour of the cultures and was particularly noticeable when using the G5 and G6 dendrimers.

To confirm if this enhanced osteogenic activity was specific to the hBMP-2 gene, as earlier suggested, the response of the osteogenesis markers in the MSC cultures after transfection with polyplexes formed by dendrimers and the β-Galactosidase reporter gene containing plasmid was also evaluated. The results obtained here did not differ from the control.

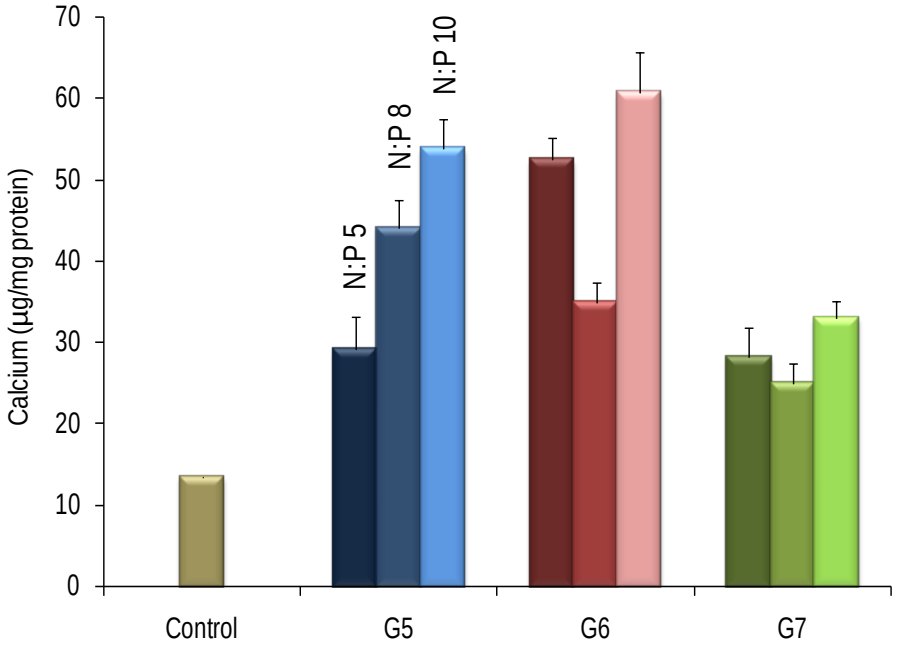

Figure 7. Calcium deposition on extracellular matrix. The cells were transfected with a plasmid DNA containing the hBMP-2 gene using generations 5, 6 and 7 of PAMAM dendrimers, and cultured for 21 days. The values represent means from three replicates ± s.e.m..

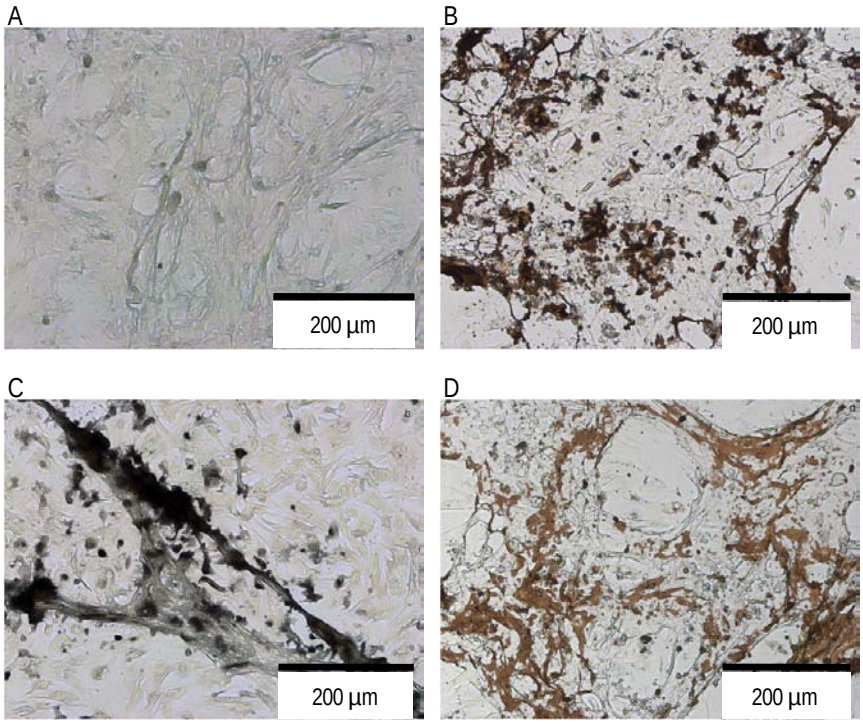

Figure 8. *Von Kossa* staining showing matrix mineralization. (A) Non transfected MSCs were compared with MSCs transfected with a plasmid DNA containing the hBMP-2 gene using PAMAM dendrimers of generations (B) 5, (C) 6 and (D) 7 at a N:P charge ratio of 10. Cells were cultured for 21 days.

4. DISCUSSION

The reported successful works[23-25] on the use of cationic dendrimers as vectors for gene delivery encouraged the present authors to assess their efficacy on MSCs with a view on applications related to bone regeneration and tissue engineering. To our knowledge, the present study is the first of its kind in this field. The efficacy of dendrimers in gene delivery can be explained by the charge-based interactions between these molecules and DNA. The PAMAM dendrimers bear positively charged primary amine groups on their surface, while having tertiary amine groups inside. The primary amine groups participate in DNA binding, compact DNA into nanoscale particles and promote the cellular uptake of DNA, while the buried tertiary amino groups act as a proton sponge in endosomes and enhance the release of DNA into the cytoplasm[21,30].

From the beginning, investigators have searched for a correlation between transfection and the molecular size and shape of the PAMAM dendrimers. Haensler and Szoka (1993) found that the generation 6 PAMAM dendrimers, with a diameter of 6.8 nm and resembling the histone core in chromatin around which DNA is wrapped, were optimal in mediating transfection[23]. Another study using a variety of cell lines showed that the best performing generation (from G5 to G10) for cell transfection was strongly dependent on the cell type, and that the DNA binding ability increased with the generation number[26,31]. However, while some authors describe PAMAM dendrimers as conducting to high levels of transfection, others still search for ways of modifying them to improve their efficiency[24]. In this respect, it is worth mentioning that partially degraded PAMAM dendrimers were shown to be 100-fold more active as gene delivery vectors[21].

In order to determine their behaviour as gene transfer agents towards MSCs, three different generations of PAMAM dendrimers (G5, G6 and G7), at different N:P charge ratios, were analysed in the first set of the present results. Plasmid DNA encoding the *β*-galactosidase reporter gene was employed here. From the data it was found that the MSCs could be transfected with the PAMAM dendrimers. However, the observed transfection levels were very low. Several investigators have faced the same problem when using other non-viral gene vehicles and MSCs, thereby indicating that this cell type, like other primary cells, is typically refractory to transfection[32]. Nevertheless, even with low levels of transfection, it was possible to distinguish

different levels of transfection efficacy amongst the different dendrimer generations and the different N:P charge ratios tested. The best results were obtained with the G5 and G6 dendrimers, using a N:P ratio of 10. These results however, corresponded to higher cytotoxicity conditions. This was to be expected, since the cytotoxicity of cationic polymers has been reported to be a function of the interaction between the polymer and the cell membrane and/or of cellular uptake efficiency, two factors that are also important for the efficacy of gene delivery agents[33,34]. In practice, the increase in transfection efficiency was accompanied by an increase in the polymer concentration. Interestingly, the cytotoxicity shown by the polyplexes was not very different from that presented by the dendrimers. This is in contrast to studies which have shown that complexation with DNA tends to reduce toxicity[20]. The present study also reinforces the conclusions reached by other authors that transfection may strongly vary with the type of cells used. Here, the PAMAM dendrimers were shown to be efficient towards the HEK 293T cell line, which was used as a positive control in the experiments. Additionally, transfection varied drastically with the cell passage number, showing that not only the cell type but also the cell proliferation state, are important parameters to take into account. Indeed, the transfection efficiency has been shown to be related with the cell cycle activity and cell growth[35,36].

It has been reported that while MSCs possess intrinsic osteogenic activity, their level of BMP production is extremely low[37]. Combined therapy with recombinant BMP has been shown to promote osteogenic differentiation *in vitro*[38-41] and to induce bone formation *in vivo*[42,43]. The high concentrations of BMP-2 protein required to achieve a biological effect, as well as its rapid degradation after introduction into the body, has however rendered its use cost ineffective. Recently, several studies on bone regeneration have reported successful results using adenoviruses, retroviruses and baculoviruses vectors to carry BMP-2 cDNA[8,10-12,43,44]. Although work in this field is expanding, the data available on nonviral gene delivery for bone regeneration and tissue engineering applications is still scarce[6,45].

In the second set of the present results, using a DNA plasmid encoding the hBMP-2 gene, it was aimed to evaluate whether or not a low transfection level would be sufficient for the envisaged application, i.e., to improve the *in vitro* differentiation of MSCs towards the osteoblastic lineage. All quantitative assays involving the osteogenic markers (ALP activity, osteocalcin and mineralization) responded positively to this hypothesis. Taken together, the results confirmed

optimal transfection efficacies for the G5 and G6 PAMAM dendrimers at a N:P charge ratio of 10 (as for the assays employing the *β*-galactosidase reporter gene). The *von Kossa* staining performed here was in agreement with the quantitative data.

In conclusion, a coherent set of results was obtained indicating a clear correlation between the data obtained with the hBMP-2 and the *β*-galactosidase reporter gene containing plasmids. Furthermore, the low levels of transfection achieved were shown to be sufficient to promote the osteogenic differentiation of MSCs. A recent *in vivo* study, using C3H10T1/2-derived C9 MSCs expressing BMP-2 under control of the doxycycline(Dox)-repressible promoter, revealed that the short-term expression of this proteinaceous growth factor was sufficient to irreversibly induce bone formation. From this it is clear that a stable genetic modification of MSCs is not required for stem cell-based bone engineering[46]. The fact that nonviral gene delivery vectors, such as polycations, only mediate the transient expression of the transferred genes, opens the door to the use of such vectors in this kind of application. In addition, the present *in vitro* results suggest that a low level of gene expression may also be enough for the successful treatment of bone defects or bone-related diseases. This finding may be relevant in the clinical scenario, where the delivery of high or uncontrollable doses of BMP-2 is a cause of concern and thus should be avoided. The present results also point out the relevance of focusing research on the development of nonviral gene delivery vectors on concrete applications, instead of focusing on the improvement of the transfection efficiency.

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CHAPTER III.

Delivery of the BMP-2 Gene into MSCs: a Biomimetic approach using RGD Nanoclusters based on Poly(amidoamine) Dendrimers

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Delivery of the BMP-2 Gene into MSCs: a Biomimetic approach using RGD Nanoclusters based on Poly(amidoamine) Dendrimers*

ABSTRACT

Poly(amidoamine) (PAMAM) dendrimers (generation 5 and 6) with amine termini were conjugated with peptides containing the amino-acid sequence arginine-glycine-aspartic acid (RGD) having in view their application as gene delivery vectors. The idea behind the work was to take advantage of the cationic nature of dendrimers and of the integrin targeting capabilities of the RGD motif. Dendrimers were used as scaffolds for RGD clustering and, by controlling the number of peptides (4, 8, and 16) linked to each dendrimer, it was possible to evaluate the effect of nanoclusters RGD density on the gene delivery process. The new vectors were characterized in respect to their ability to neutralize, compact and dissociate from plasmid DNA (pDNA). The complexes formed by the vectors and pDNA were studied concerning their capacity of being internalized by cells and of transferring genes. Transfection efficiency was analysed, first, by using a pDNA encoding for Enhanced Green Fluorescent Protein and Firefly Luciferase and, second, by using a pDNA encoding for Bone Morphogenetic Protein-2 (BMP-2). Gene expression in mesenchymal stem cells (MSCs), a cell type with relevance in the regenerative medicine clinical context, was enhanced using the new vectors in comparison to native dendrimers and was shown to be dependent on both the electrostatic interaction established between the dendrimer moiety and the cell surface and the nanoclusters RGD density. The use of dendrimer scaffolds for RGD cluster formation is a new approach that can be extended beyond gene delivery applications. These nanoclusters can be connected with other materials and systems where RGD clustering is important for modulating cellular responses.

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[#] Equally contributing authors

1. INTRODUCTION

The aminoacid sequence arginine-glycine-aspartic acid (Arg-Gly-Asp, RGD), together with integrin receptors in cell surfaces, provide a major recognition system that can be used as a tool in numerous biomedical applications. Since the discovery of RGD in fibronectin, in 1984[1], and its central role in cell adhesion[2,3], the RGD sequence has been applied in the design of new drugs and materials with the aim of controlling their behaviour and improve their efficacy. In the last few years, the RGD motif has been explored as a cell-targeting agent in anti-tumour therapies, cell imaging, and nucleic acids and drug delivery, as well as a mean to modulate the process of integrin regulation and increase the adhesion of cells to substrates in the Biomaterials and Tissue Engineering fields[4-17].

Integrins constitute a family of cell-adhesion receptors which simultaneously interact with the proteins in the extracellular matrix and the internal constituents of the cell[18,19]. Integrins transfer signals bidirectionaly so that the cell can sense and respond to its environment. They consist of two distinct subunits (noncovalent heterodimers α and β), each containing a transmembrane domain, a large extracellular domain and a small cytoplasmic domain. Many integrins respond to RGD-binding and changes in cell morphology, differentiation, proliferation and gene expression consequently occurs. Interestingly, RGD peptide clustering has been shown to interfere with cell responses and with the gene transfer properties of non-viral vectors[10,11,13,16]. Additionally, clustered RGD ligands present in the penton base capsomers at the capsid icosahedral vertices of several adenovirus types were shown to have a role in promoting virus entry into host cells[20,21]. Integrin-mediated gene delivery together with RGD ligands clustering can, thus, be a suitable biomimetic approach to be used in non-viral gene delivery.

Dendrimers were shown to be very attractive as non-viral gene delivery vectors[22-25]. They are tree-like molecules that growth from a central core, are composed of several layers (called generations) and present a multivalent surface. In particular, poly(amidoamine) (PAMAM) dendrimers with amine termini (they are cationic molecules at physiological pH) are the most studied dendrimers for gene delivery and were shown to present an intrinsic ability to bind, compact and deliver pDNA into a high variety of cells[22-25]. Partially degraded PAMAM dendrimers are even available in the market (under the names Superfect® and Polyfect®) and are frequently

used as reference materials for non-viral gene delivery[26,27]. The success of PAMAM dendrimers in gene delivery has been attributed to a "proton-sponge" effect[24] as, in addition to the primary amines at the surface, they possess secondary and tertiary amines in their interior. That is, dendrimers act like a buffer system inside endosomes or endo-lysosomes (containing digestive enzymes) leading to the pumping of protons and concomitant influx of chloride ions. The ionic strength inside these organelles will then increase causing their osmotic rupture and the release into the cytoplasm of the pDNA or the complexes formed between dendrimers and pDNA.

The efficacy of PAMAM dendrimers as gene transfer agents, however, greatly varies with the cell type[28]. Recently, we showed that PAMAM dendrimers (without any treatment or functionalization) were able to deliver the BMP-2 gene to mesenchymal stem cells (MSCs) but with a low efficiency[29]. In spite of *in vitro* osteogenic differentiation of MSCs achieved as shown by several osteogenic markers, there is an unquestionable interest in raising the transfection efficiency when using these cells due to their promising properties and clinical relevance in several fields. Indeed, MSCs isolated from several adult tissues, show immune suppressive properties, and are considered multipotent, being of key importance in tissue engineering and regeneration[30,31].

Herein we designed a new family of gene delivery vectors based on PAMAM dendrimers conjugated with RGD containing peptides (GRGDSPC). In fact, MSCs were shown to express the integrin receptors $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, $\alpha_6\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ at their surfaces[32,33] which are known for recognizing the RGD sequence[3]. The idea subjacent to the work was to take advantage of the cationic nature and the "proton-sponge" effect of these dendrimers and of the RGD moiety targeting capabilities. The multivalent surface of dendrimers makes them ideal scaffolds for RGD containing peptides attachment and, by varying the degree of functionalization, the effect of RGD peptide targeting and clustering can be evaluated in the process of gene delivery. Gene delivery vectors were prepared by conjugating generation 5 (G5) and generation 6 (G6) of PAMAM dendrimers with 4, 8 and 16 peptide units. The complexes formed between RGD modified dendrimers and pDNA were characterized and their cytotoxicity evaluated. Transfection was, in a first step, evaluated using a plasmid encoding for Enhanced Green Fluorescent Protein (EGFP) and Firefly Luciferase (Luc) and, further, using a plasmid encoding for BMP-2.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Generation 5 and 6 PAMAM dendrimers, possessing ethylenediamine cores and amine *termini*, were purchased in water solution from Dendritech Inc. (USA). The molecular weights and number of amine surface groups presented by the dendrimers were 28,826 u.m.a. and 128, and 58,048 u.m.a. and 256, respectively, for G5 and G6. The peptide GRGDSPC was purchased from American Peptide Company, Inc. (USA) with a purity of 95 %. Tissue culture plates were from NUNC. Agarose was purchased from Cambrex Bio Science Rockland (USA) and N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) i.e. HEPES from USB (USA). The pDNA encoding for enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) with a cytomegalovirus promoter (CMV) was generously provided by Prof. Tatiana Segura (Dep. of Chemical and Biomolecular Engineering, UCLA, USA). The pDNA encoding a human BMP-2 sequence inserted downstream the CMV promoter (pcDNA3.1/Hist©/hBMP2, 8.5 kb) was kindly donated by Professor Yasuhiko Tabata, University of Kyoto, Japan). All other materials and reagents were obtained from SIGMA-ALDRICH unless otherwise stated.

2.2. Experimental determination of primary amine group content of dendrimers

The primary amine group content of G5 PAMAM dendrimers was determined by spectrophotometry after reaction of the free amine groups with 2,4,6-trinitrobenzenesulphonic acid (TNBS), as described in the literature[34], and using glycine as standard. A total of 115 and 220 amine groups were attributed to G5 and G6 dendrimers, respectively, being these values in agreement with the Mass Spectrometry data furnished by Dendritech Inc. Based on this result, the TNBS method was further used to calculate the concentration of dendrimers (also of functionalized dendrimers) when needed. In both cases the standard solutions and the sample solutions were serially diluted in 0.1 M sodium tetraborate to a final volume of 1 ml. To each standard and sample 25 µl of TNBS (0.03 M) diluted in water was added. After 15 min at room temperature (RT), absorption was measured at 420 nm in a GBC-Cintra 40, UV-Visible spectrophotometer.

2.3. Synthesis and characterization of dendrimer/RGD conjugates

Same experimental procedure was followed for the synthesis of G5 as well as G6 dendrimer/RGD conjugates. In a first step, G5/G6 PAMAM dendrimers (Figure 1, compound 1, C1) were substituted with various amounts of the bifunctional 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), as described earlier by Szoka *et al*.[35]. G5/G6 PAMAM dendrimers (0.50 μmol) in 2 ml buffer (0.25 M NaCl, 0.1 M phosphate, pH 8) were mixed with SPDP (2-16 μmol) dissolved in 250 μl ethanol. The mixtures were allowed to react for 3 h, at RT, under nitrogen and with continuous stirring. Several derivatives were obtained (compound 2, C2) by varying the molar ratio dendrimer/pyridyldithiol (PDP) (1/4, 1/8 and 1/16). Afterwards, low molecular weight products were removed by gel permeation chromatography on a PD-10 column (GE Healthcare) equilibrated in 0.15 M NaCl, 0.1 M phosphate buffer, pH 7.4. The fractions containing dendrimers linked to PDP were pooled, concentrated, and snap frozen in liquid nitrogen and stored at -80 ºC. The dendrimer content in C2 was estimated by the 2,4,6-trinitrobenzenesulphonic acid (TNBS assay as described previously. The degree of modification with the SPDP linker was evaluated spectrophotometrically at 343 nm by the release of pyridine-2-thion (compound 4, C4) after reduction of an aliquot with excess dithiothreitol (DTT, 0.1 M). As calibration standards, known concentrations of SPDP solutions were treated with 0.1 M DTT under the same conditions, and the changes in absorbance were used as a measure of pyridine-2-thion (C4) in solution. In a second step, the GRGDSPC peptides with a terminal cysteine were dissolved in 100 mM phosphate buffer, pH 7.2 and mixed, under nitrogen, with C2 compounds diluted in 100 mM phosphate buffer, pH 7.2. An excess of peptide (2-fold) to PDP present in C2 was used to increase reaction yields since a competitive peptide dimerization reaction may occur. After 3 h at RT, the amount of released pyridine-2-thion (C4) was measured at 343 nm to determine the extent of reaction. Peptide-functionalized dendrimers (C5) were purified by gel filtration using PD-10 columns equilibrated in 0.5 M NaCl, 20 mM sodium acetate, pH 5. Conjugates were applied to the column and the void fractions containing the conjugates were dialyzed against phosphate buffered saline (PBS) solution, pH 7.2, using dialysis tubes with a molecular weight cut-off of 10 kDa (Spectrum Laboratories) for 3 days. After dialysis, conjugates were sterile filtered and aliquots were snap frozen in liquid nitrogen and stored at -80ºC. Conjugates were characterized by ^{1}H NMR using D_2O as solvent in a Bruker 400 MHz Avance II^{+} NMR spectrometer.

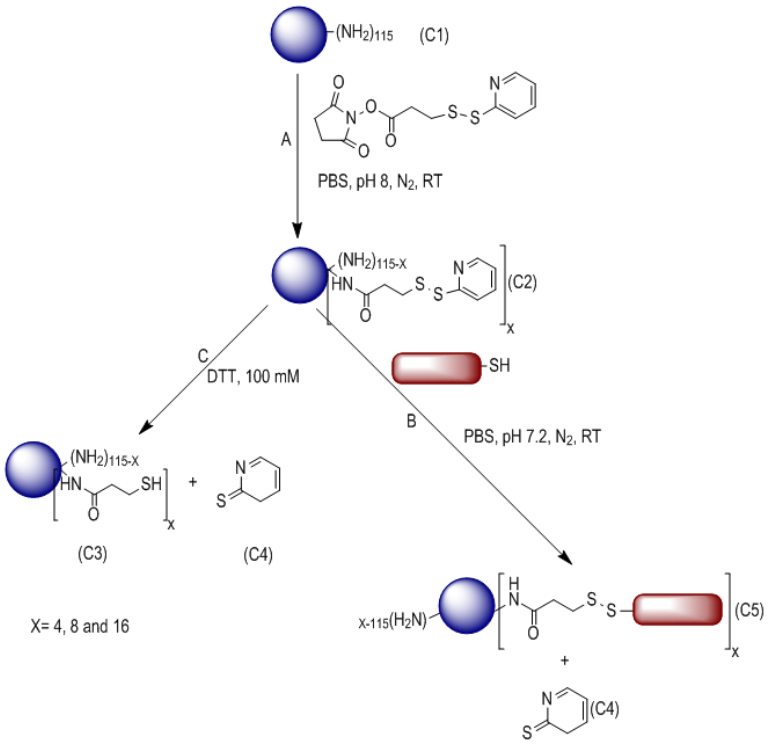


Figure 1. Schematic illustration of the synthesis of PAMAM dendrimers/RGD conjugates (pathway A+B), and of the indirect estimation of the number of peptide units by spectrophotometry (pathway A+B+C). Note: The amine content of G5 PAMAM dendrimers (115 amines/dendrimer) was previously determined experimentally.

2.4. Plasmid DNA amplification

The plasmids were amplified in *Escherichia Coli* host strain, DH5α, grown overnight in Luria-Broth Base medium containing antibiotic. Afterwards, the plasmids were isolated and purified using a GenElute™ HP Endotoxin-Free Plasmid Megaprep Kit. The isolated plasmids were dissolved in a small amount of ultra-pure water and the quantity and quality of the purified plasmid DNA was assessed by spectrophotometric analysis at 260 and 280 nm. The plasmid integrity was assessed through agarose gel electrophoresis. pDNA was stored in ultrapure water at -20ºC.

2.5. Complex assembly

The complexes formed by pDNA and dendrimers/RGD conjugates were prepared at several N:P ratios by mixing equal volumes of the conjugate solution and the pDNA solution. Conjugate and pDNA concentrations varied according the experiments. Hepes-buffered glucose (HBG, Hepes 10 mM, Glucose 5% w/w, pH = 7.1) was always used to prepare these solutions. Polyplex solutions were vortexed gently and allowed to incubate for 20 min at room temperature prior to experiments. Native PAMAM dendrimers were always used as a control. Superfect® (Qiagen, Germany) was also used as a reference. Superfect polyplexes were prepared according to the manufacturer's instructions for transfection of primary cells.

2.6. Agarose gel electrophoresis retardation assay

Gel electrophoresis in agarose gels was carried out at 75 V. Agarose gel (0.7 % w/v) containing ethidium bromide (0.05 µl.ml^{-1}) was prepared in Tris-acetate-EDTA buffer. Complex solutions were prepared at different N:P ratios, as described above, using 1 µg of pDNA diluted in 50 µl of buffer and 2.5 µl of Blue Loading Buffer (Fermentas, Germany) were added before the samples were subjected to gel electrophoresis. Vectors-pDNA interaction is shown by a lack of migration of the pDNA in the electrophoretic field. DNA was visualized by a UV lamp using a BioRad Imaging System.

2.7. Pico green assay for evaluation of pDNA condensation

The complexes formed by pDNA and dendrimer/RGD conjugates were prepared at different N:P ratios, as described above, with 0.1 µg of pDNA in 100 µl of HBG, and the mixtures were incubated for 20 min at room temperature. Then, the complexes (100 µl) were transferred into white flat-bottom 96-well plates followed by addition of 100 µl Picogreen (PG) reagent diluted 200× in Tris-EDTA buffer (TE, 10 mM Tris, 1 mM EDTA, pH 7.5) and incubated further for 5 min. Each measurement was performed in triplicate and the fluorescence of PG (λ_{ex}= 485 nm, λ_{em}= 535 nm) for each sample was measured using a microplate reader (model Victor^3 1420,

PerkinElmer). The relative fluorescence percentage (%F) was determined using the equation below:

$$\%F = 100 \times \frac{F_{sample} - F_{blank}}{F_{DNAonly} - F_{blank}}$$

2.8. Pico green assay for evaluation of salt-induced complex dissociation

The complexes formed by pDNA and dendrimer/RGD conjugates were prepared as described above with N:P ratio of 5 and diluted with water to a concentration of 0.3 μg pDNA/100 μl solution. PG solutions were prepared by a 200× dilution of PG reagent with 10mM Hepes buffer containing adequate concentrations of NaCl. After the complex solutions were arrayed into white flat-bottom 96-well plates, 100 μl of the PG solutions were added to each well to achieve the desired concentration of NaCl (0.1 M, 0.3 M, 0.5 M, 0.7 M and 1.5 M). The fluorescence of Picogreen for each sample was then measured.

2.9. Isolation and culture of rat bone marrow-derived mesenchymal stem cells

Rat bone marrow-derived mesenchymal stem cells (MSCs) were isolated from long bones of 8-week-old male Wistar rats (Charles River Laboratories, Spain). Following euthanasia by pentobarbital 20% (v/v), femora were aseptically excised, cleaned of soft tissue, and washed in PBS. The metaphyseal ends were cut off and the marrow was flushed out from the midshaft with 5 ml of α-Minimum Essential Medium (α-MEM) using a 23-Gauge needle and syringe. The cells were centrifuged (600 g, 10 min), suspended in fresh medium containing 10% heat-inactivated foetal bovine serum (FBS, Gibco), 50 $\mu g.ml^{-1}$ ascorbic acid, 100 $U.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ of streptomycin - the basic medium - and seeded in 75 cm^2 flasks. Cells were cultured at 37°C in a 5% CO_2 atmosphere. After removal of non-adherent cells and medium exchange at day 3, cells were harvested at day 7 by trypsinization, and used in subsequent experiments.

2.10. Cellular uptake studies by fluorescence-activated cell sorting (FACS)

Cells were seeded in 12-well plates at a density of 1.5×10^4 cell.cm^{-2} and incubated in basic medium at 37ºC, 5% CO_2, for 24 h, to yield a cell confluency of around 60-80%. Prior to complex formation, pDNA was labelled with PicoGreen dye (Invitrogen, Carlsbad, CA) according to the manufacturer's directions. Complex solutions (100 µl) were prepared at a N:P ratio of 5 as previously described. Cells in 1 ml of basic medium were then transfected using 100 µl of the complex solution (2 µg.cm^{-2} of pDNA was used). The cells were incubated with each solution for 1 h and then rinsed twice with PBS. The extracellular fluorescence associated with cell surface-bound nanoparticles was quenched with 0.4% (w/v) Trypan-Blue (TB) for 5 min. The cells were washed multiple times with PBS, trypsinized, pelleted, and resuspended in 400 µl of PBS containing 2% FBS for FACS analysis (Cytomics FC500, Beckman Coulter). The untreated cells were used as a control to set up the gating and to adjust the forward and side scatter and the cellular debris were gated out of the sample. A total of 10000 events within the gated region were collected in triplicate for each sample. The positive fluorescence level was established by visual inspection of the histogram of negative control cells such that less than 1% appeared in the positive region using PG-labelled naked pDNA transfected cells as the primary negative control. Data was analysed using CXP software analysis program.

2.11. Gene delivery studies

Gene delivery was studied based on reporter gene expression (using pDNA encoding EGFP and Luc) and also on BMP-2 expression (using pDNA encoding BMP-2).

Expression of the Luc gene: Cells were seeded at 1.5×10^4 cell.cm^{-2}, in 24-well plates, 24 h prior to transfection. At the time of transfection, cells reached 60-80% confluency. Before contact with polyplexes, medium was exchanged for 0.5 ml of fresh basic medium. Complex solutions (100 µl) were then added to the cells and, after 4 h, the culture medium was again replaced with fresh medium. Transfection was carried out at a N:P ratio of 4 and using 2 µg.cm^{-2} pDNA. Non-transfected cells were used as negative controls. At different time points (48, 72 and 96 h) after transfection, the media was removed and the cells were washed with PBS solution and treated with 100 µl reporter lysis buffer (Promega). Cell lysates were analysed for luciferase activity with

Promega's luciferase assay reagent in triplicate (following the supplier's instructions). For each sample, the microplate reader (model Victor^3 1420, PerkinElmer) was set for 3 s delay with signal integration for 10 s. The amount of protein in cell lysates was determined using the bicinchoninic acid assay (BCA assay) with bovine serum albumin as a standard. The gene delivery efficiency of each sample was characterized by Firefly Luciferase expression and denoted as relative light units per mg of protein (RLU.mg^{-1} protein).

Expression of the EGFP gene: Enhanced Green Fluorescent protein expression studies were carried out as mentioned above for the Luc gene expression. Twenty-four hours after transfection, cells were observed with an inverted fluorescence microscope (Nikon Eclipse TE 2000E) equipped with a cold Nikon camera. Digital image recording and image analysis were performed with the NIS Elements Advanced Research (version 2.31) software.

2.12. Cytotoxicity studies

The cytotoxicity of the gene delivery vectors (alone) and the respective complexes they form with pDNA were studied. Cytotoxicity was evaluated by determining the percentage of cell viability (in respect to unexposed cells) using the resazurin reduction assay that establishes a correlation between the cellular metabolic activity and the number of viable cells in culture[36].

Gene delivery vector cytotoxicity: Cell viability was studied as a function of the gene delivery vector type and concentration. Cells were seeded in 96 well-FluoroNunc plates at a density of 3x10^4 cell.cm^{-2}. After 24 h, medium was replaced with fresh basic medium and 10 µl of each polymer (diluted in HBG) was added to achieve the final desired concentration. After 4 h, the medium was exchanged for fresh medium containing 0.1 mg.ml^{-1} resazurin and incubated for another 4 h. Resorufin fluorescence (λ_{ex} = 530 nm, λ_{em} = 590 nm) was measured in a microplate reader (model Victor^3 1420, PerkinElmer).

Complex cytotoxicity: The information about complex cytotoxicity was obtained during the experiments performed to study the expression of the Luc gene (described above), being cell viability evaluated 24 h post-transfection.

2.13. Fluorescence microscopy studies

Fluorescence microscopy was used to visualize pDNA cellular uptake. Prior to complex assembly, pDNA was labelled with rhodamine isothiocyanate (RITC) by a slight modification of a reported method[37]. Briefly, 0.1 mg of pDNA diluted in 297 μl of sodium carbonate-buffered solution (0.1 M, pH 9.0) was mixed with 3 μl of RITC solution (100 mM solution prepared in dimethyl sulfoxide) at RT, for 5 h. The RITC-labelled pDNA was separated from residual RITC by gel filtration using a PD 10 column (GE Healthcare), followed by ethanol precipitation to obtain the RITC-labelled pDNA. Experiments were performed in the same conditions as above described for transfection experiments although using RITC-labelled pDNA at a lower concentration (1 μg.cm^{-2}) to avoid blur. Live cells were analyzed under the inverted fluorescence microscope (Nikon Eclipse TE 2000E) four hours post transfection after quenching of extracellular fluorescence with 0.4 % (w/v) TB for 5 min and washing with PBS. The RITC-labelled pDNA was detected by a red signal.

For observation at higher magnifications, 24 h prior to transfection, cells were seeded at a density of 1.5×10^4 cell.cm^{-2} in 24-well plates containing collagen-treated cover slips. Before contact with complexes, medium was exchanged for fresh basic medium. Transfection was carried out with complexes prepared at N:P ratio of 5 using 1 μg.cm^{-2} RITC-labeled pDNA. The distribution of pDNA inside cells was analyzed 2, 4, and 24 h post-transfection. Thirty minutes prior, the acidic late endosome and lysosome compartments were stained with the addition of LysoSensor DND-189 dye (Molecular Probes) to the medium at a final concentration of 1 μM. After quenching with 0.4% (w/v) TB, cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde prepared in PBS at room temperature for 10 min. Cell nuclei were then stained using a 300 nM 4',6-diamidino-2-phenylindole (DAPI) solution for another 10 min. Cells were washed several times with PBS and stored at 4ºC (protected from light). Fluorescence images were acquired using the Nikon Eclipse TE 2000E inverted microscope equipped with a 100x NA 0.5-1.3 Plan Fluor objective.

2.14. Statistics

Statistical analyses were performed using GraphPad Prism 5.0 for Windows. Results are reported as mean± standard error of mean (s.e.m). Unpaired, Student's t-test and 2-way ANOVA with Bonferroni Post Hoc test were used to assess the statistical differences between the group means.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of PAMAM dendrimers/RGD conjugates

Generation 5 (G5) and generation 6 (G6) PAMAM dendrimers with amine termini (C1) were conjugated to GRGDSPC peptides via a two-step method using the 3-(2-pyridyldithio) propionic acid N-hydroxy-succinimide ester (SPDP), a heterobifunctional crosslinker which contains both amine- and sulfhydryl-reactive groups (Figure 1). For linkage, a cysteine residue was present at the end of the amino-acid sequence. During the synthesis, the concentration of dendrimers (also of functionalized dendrimers) was determined by spectrophotometry, based on primary amine content. The degree of functionalization was under stoichiometric control and was indirectly estimated by spectrophotometric quantification of pyridine-2-thion (C4) that is released after reduction of the dendrimer-PDP intermediate (C2) with excess of dithiothreitol (DTT) and after reaction of C2 with the peptides. Conjugates with approximately four (RGD4-G5 and RGD4-G6), eight (RGD8-G5 and RGD8-G6) and sixteen (RGD16-G5 and RGD16-G6) peptide arms were prepared. Table 1 shows the average number of PDP moieties per dendrimer determined after reaction A and the average number of peptides per dendrimer after reaction B.

The conjugates were further characterized by ^{1}H NMR spectroscopy (400MHz) using D_2O as solvent (as exemplified in Figure 2). ^{1}H NMR spectra revealed the solvent peak of D_2O at 4.7 ppm and multiple peaks attributed to PAMAM dendrimers between 2.2 and 3.4 ppm. The successful conjugation of RGD peptides to PAMAM dendrimers is showed by the appearance of new proton signals such as the methylene protons around δ = 3.9 (CH_2, s) from glycine aminoacid and methine protons (CH, s) at δ = 3.65 from arginine aminoacid.

Table 1. Average number of PDP and of peptides per dendrimer, respectively, after reactions A and B. *Theoretical value based on the molecular weight of dendrimers provided by the supplier.

Conjugate	Average number of PDP per dendrimer (after reaction A)	Average number of peptides per dendrimer (after reaction B)	Mol. weight* (u.m.a)
RGD4-G5	4.4	4.2	30412.9
RGD8-G5	8.2	8.2	33520.6
RGD16-G5	18.8	17.5	40745.8
RGD4-G6	4.6	3.9	53902.9
RGD8-G6	8.3	7.2	56466.6
RGD16-G6	16.9	14.4	62060.4

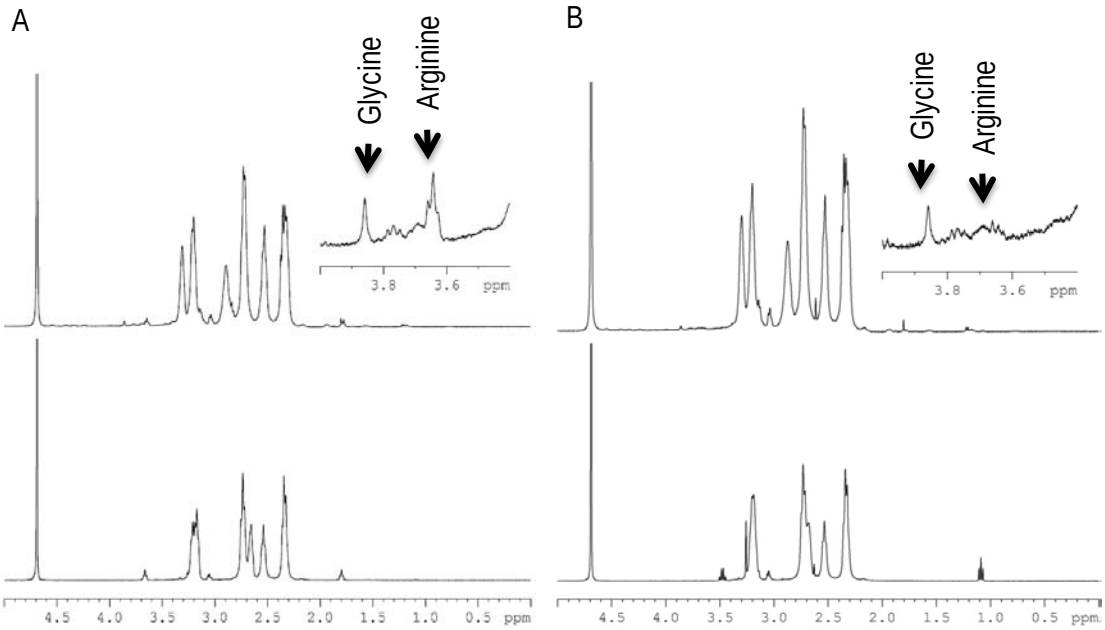


Figure 2. ^{1}H NMR spectra (400MHz, in D_2O) of (A) G5 PAMAM/RGD conjugate (sixteen peptides per dendrimer) and native G5 PAMAM dendrimer, inset is a magnified image of part of the spectrum from 3.4-4.0 ppm; (B) G6 PAMAM/RGD conjugate (sixteen peptides per dendrimer) and native G6 PAMAM dendrimer, inset is a magnified image of part of the spectrum from 3.4-4.0 ppm.

3.2. Characterization of the complexes formed by PAMAM dendrimers/RGD conjugates and pDNA

PAMAM dendrimers/RGD conjugates were first investigated for their ability to bind, neutralize and compact pDNA. Plasmid DNA encoding for enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) was used in these studies. Prior to all studies, conjugates were mixed with pDNA at several N:P ratios in HBG buffer for complex formation. Agarose gel retardation assays revealed that binding to and charge neutralization of pDNA occurred at N:P ratios of 1 for all the vectors. These results are exemplified in Figure 3 for G5 PAMAM dendrimer and for its conjugates. By using the PicoGreen (PG) dye (Figure 4), we concluded that complete pDNA packaging was achieved at an N:P ratio of 2 for all vectors. Indeed, PicoGreen gives a fluorescent signal when bounded to dsDNA. In these experiments, complexes are first prepared and then allowed to be in contact with PicoGreen; only free pDNA will bind with PicoGreen and give rise to a fluorescent signal. No fluorescence signal is indicative of all pDNA compacted.

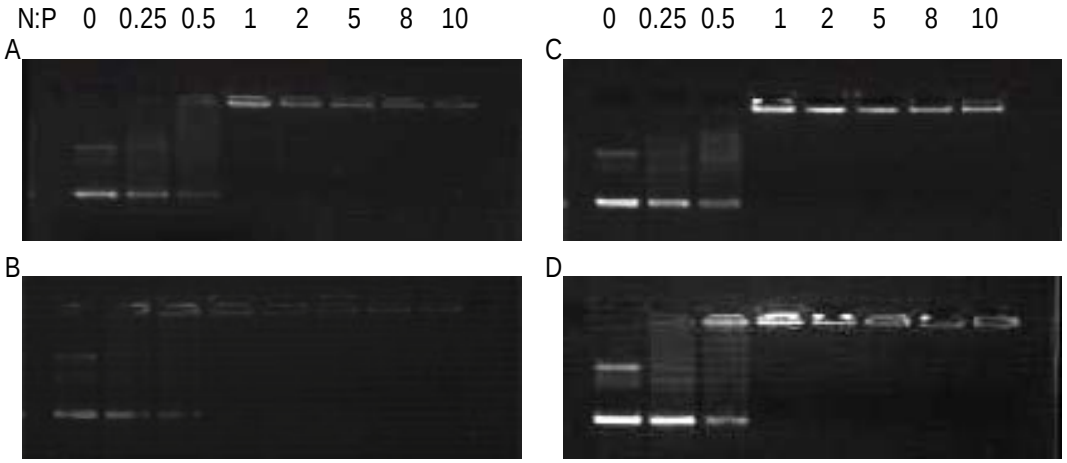


Figure 3. Electrophoretic pattern of pDNA complexes with G5 (A), RGD4-G5 (B), RGD8-G5 (C) and RGD16-G5 (D) vectors. Lane 1, pDNA only; Lane 2-8, for an N:P ratio of 0.25, 0.5, 1, 2, 5, 8 and 10, respectively.

pDNA packaging is essential for its entry inside cells but, after, its dissociation from the vector is a crucial step for an efficient gene delivery process. The evaluation of the strength of the interaction between pDNA and the gene carrier is, thus, an important parameter that may provide clues concerning the mechanism of gene transfer. Complexes formed by pDNA and the vectors were then submitted to an increasing ionic strength by increasing the concentration of sodium

chloride (NaCl) in solution. PicoGreen assay was used to measure the amount of pDNA released at each NaCl concentration. Results are presented in Figure 5 and do not show significant differences either between G5 and G6 dendrimer based vectors or between native dendrimers and dendrimers/RGD conjugates. Release of pDNA occurred by increasing the ionic strength in solution but the percentage of unpacked pDNA did not surpassed 25%. Results indicate the existence of a strong electrostatic interaction between pDNA and the gene delivery vectors that is likely related with the high cationic character of the dendrimer moiety.

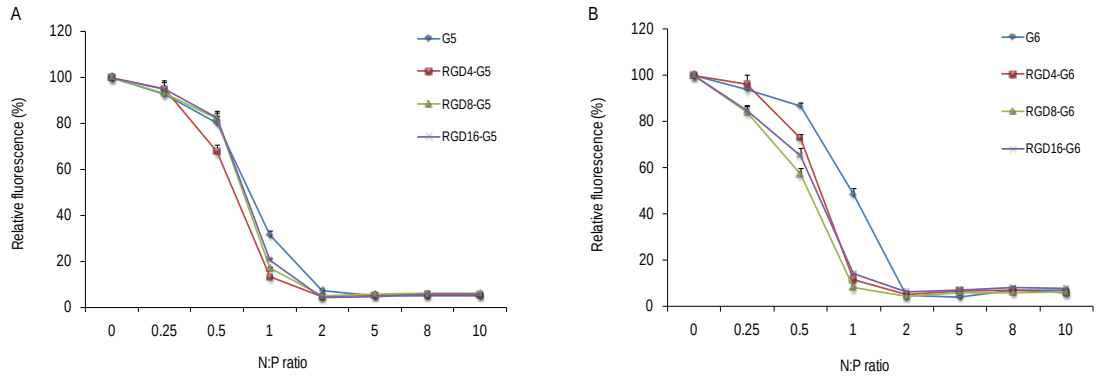


Figure 4. PicoGreen (PG) assay results at various N:P ratios for (A) G5 and G5 PAMAM dendrimers/RGD conjugates and (B) G6 and G6 PAMAM dendrimers/RGD conjugates. The results are reported as the relative percentage of PG fluorescence, where 100% intensity was observed for a N:P of 0 (pDNA only). Data represents the mean ± s.e.m., n=3.

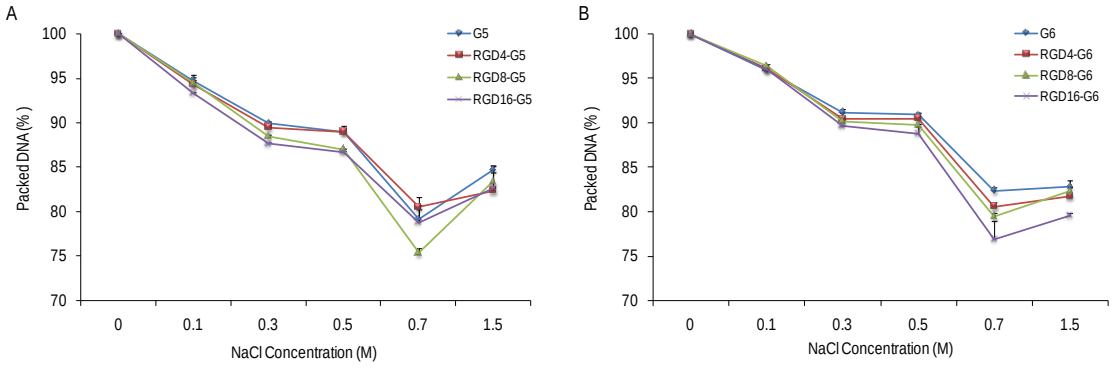


Figure 5. Salt induced dissociation of the complexes formed by pDNA and (A) G5 and G5 PAMAM dendrimer/RGD conjugates and (B) G6 and G6 PAMAM dendrimers/RGD conjugates. The PicoGreen assay was used to estimate the amount of pDNA released. Results are expressed as the mean ± s.e.m., n=3.

3.3. Cellular uptake of the complexes formed by pDNA and PAMAM dendrimers/RGD conjugates

PicoGreen-labeled pDNA was used to compare complexes uptake by MSCs through the FACS technique. Cellular uptake was studied in the presence of serum, at a N:P ratio of 5 and using a seeding density of $1.5x10^4$ cell.cm^{-2} and 1 μg.cm^{-2} pDNA (Figure 6). After 1h of contact between complexes and cells, the percentage of cells positive for PG-labeled pDNA significantly increased for dendrimers/RGD conjugates in comparison with native dendrimers ($p< 0.05$), being higher for G6 dendrimer based vectors ($p<0.05$). Maximum values were attained with dendrimers functionalized with eight peptide arms (an increase of about 20% and 10% of positive cells in respect to native dendrimers was observed when using RGD8-G5 and RGD8-G6 conjugates, respectively). The number of positive cells for PG-labeled pDNA obtained with conjugates containing 4 and 16 peptide units was similar and lower than that observed for conjugates with 8 peptide units. A slight increase in the amount of pDNA delivered per cell was also observed for functionalized dendrimers in comparison with native dendrimers. These results followed the same trend displayed by the number of cells.positive for PG-labeled pDNA.

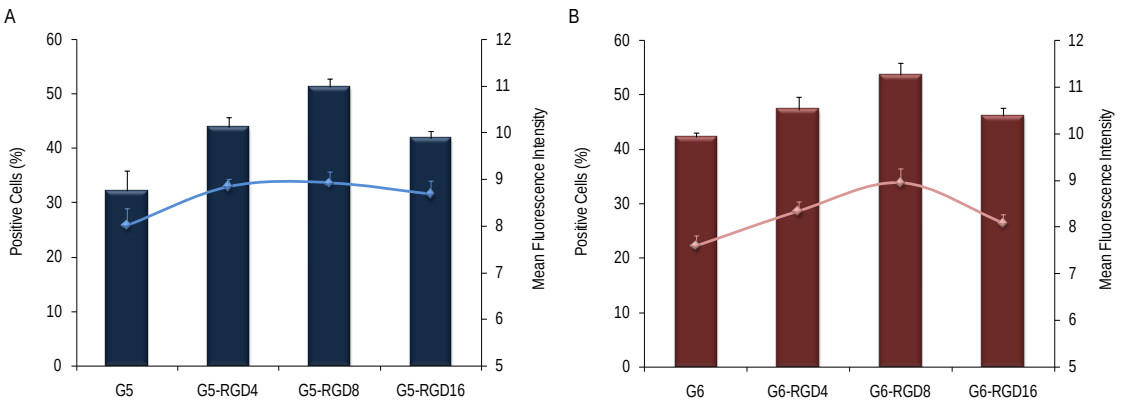


Figure 6. MSCs uptake of the complexes formed by pDNA and (A) G5 and G5 PAMAM dendrimer/RGD conjugates and (B) G6 and G6 PAMAM dendrimers/RGD conjugates, after 1 h of contact . The bars shows the number of cells positive for PG-labeled pDNA, whereas the lines reveal the amount of pDNA delivered per cell (displayed as the mean average fluorescence intensity). Results are expressed as the mean ± s.e.m., n=3.

A possible explanation for our results is that there are two possible different mechanisms by which complexes are internalized by MSCs. In one hand, complexes may enter cells through the RGD-integrin recognition system. By increasing the number of peptide arms in the dendrimer,

a higher density of RGD ligands will be displayed in the nanoclusters and this will facilitate the cellular internalization of the complex. In the other hand, cell entry will also depend on the cationic charge associated with the complex that, in this case, will be internalized following the classical endocytic pathway. By increasing or decreasing the charge of the complex, its internalization will increase or decrease, respectively. This is evident in two situations. (A) In the first one, a decrease in the number of cells positive for PG-labeled pDNA is observed when using dendrimers functionalized with 16 peptide arms. Likely, peptides can present a shield effect over the dendrimer moiety. This is in agreement with other studies made in our laboratory where the functionalization of PAMAM dendrimers with osteotropic peptides[38] and alkyl groups[39] with the aim of improving the gene delivery performance of dendrimers was performed. Other authors also report the same shielding effect[40]. In addition, by increasing the number of peptides connected to the dendrimer, the number of anionic aspartic acid residues in the periphery of the dendrimer will also increase thus reducing the net positive charge of the complexes, diminishing the complex attraction by the anionic cell surface. (B) In the second one, a higher number of cells positive for PG-labeled pDNA are observed for G6 dendrimer based vectors in comparison to G5 dendrimer based ones. This occurs because G5 dendrimer is smaller than G6 dendrimer and, so, the shielding effect caused by the peptides will be more pronounced in the first case. Cell entry will then be favored for complexes formed by pDNA and G6 dendrimer based vectors as for them the electrostatic interaction with the cell membrane will be stronger. The higher shielding effect over G5 dendrimers will result in weaker electrostatic interactions with cells, being the effect of RGD presence in cell uptake more prominent. Complex cellular uptake is, thus, a result of the combination of these two mechanisms of cell entry (one specific and the other non-specific). Cellular uptake studies were also performed using fluorescence microscopy. Figure 7 shows the merged bright field and fluorescent microscopy images of MSCs four hours after transfection with RITC-labeled pDNA (red). Although these experiments provide only qualitative data, it can be seen that there is a correlation between them and the previous quantitative data.

Figure 8 correspond to images taken at a higher magnification and shows the distribution of RITC-labeled pDNA (red) inside cells after transfection using native dendrimers and dendrimers conjugated with 8 RGD peptides. The acidic late endosome and lysosome compartments were stained with LysoSensor Green DND-189 (green), and the nucleus with DAPI (blue).

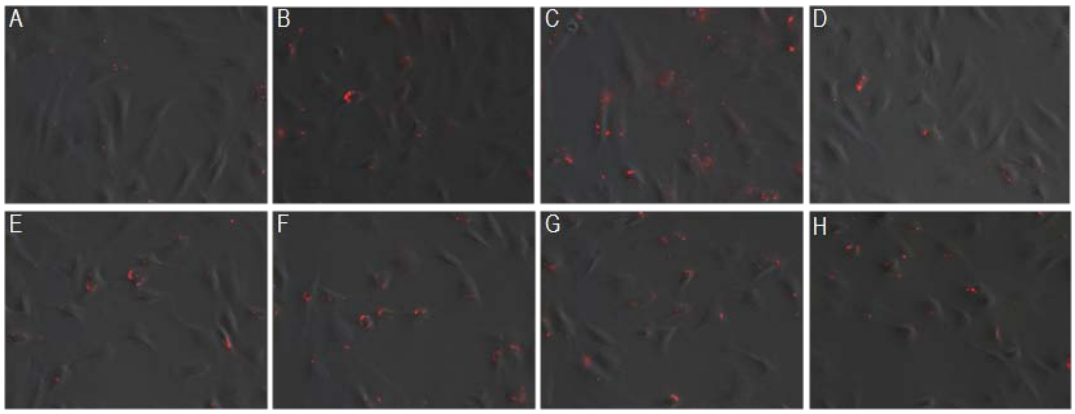
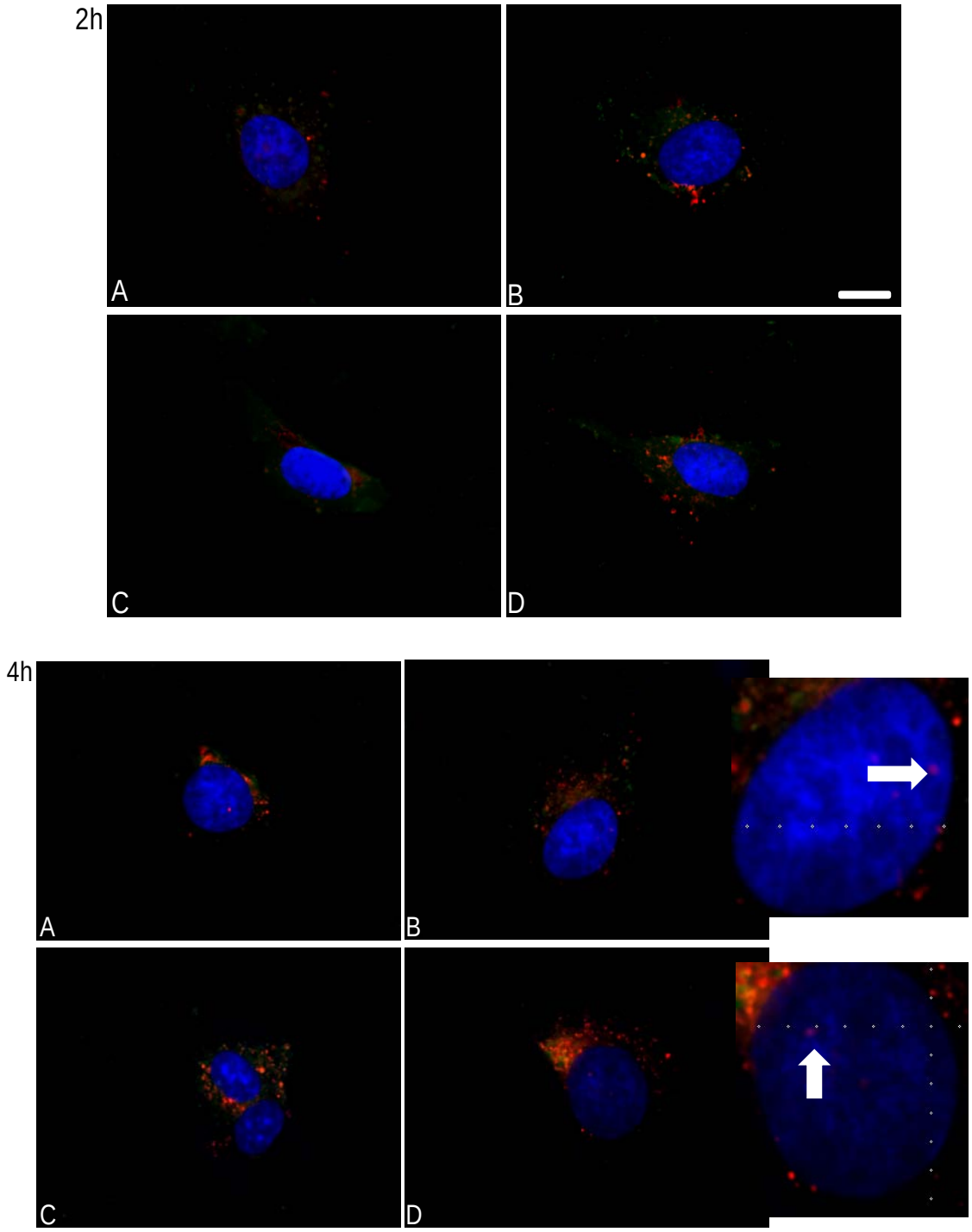


Figure 7. Microscopy RITC-labeled pDNA visualization in culture, 4 h post-transfection. Live cell images were captured. (A), (B), (C) and (D) are, respectively, the merged images of bright field mode (gray) and fluorescence mode using G5, RGD4-G5, RGD8-G5 and RGD16-G5 vectors. (E), (F), (G) and (H) are, respectively, the merged images of bright field mode (gray) and fluorescence mode using G6, RGD4-G6, RGD8-G6 and RGD16-G6 vectors. The micrographs were obtained at an original magnification of 200×.

Two hours post-transfection, a higher accumulation of pDNA could be observed inside cells when using dendrimer/RGD conjugates, being these data in agreement with FACS results which showed that they were able to deliver a high amount of pDNA per cell. As time progressed, it was possible to observe the appearance of orange spots representing the co-localization of pDNA and endosome/lysosome compartments. pDNA co-localization with the nucleus (pink dots, white arrows) could already be observed after 4 h when dendrimer/RGD conjugates were used. The number of pink dots inside the nucleus is, however, very small in all cases, whereas a high accumulation of RITC-labeled pDNA is clearly seen in nucleus periphery, in great part associated with the endosome/lysosome compartments. These microscopy results evidence a noticeable difficulty of pDNA to reach the cell nucleus where the expression of the carried genes occurs. This happens for different reasons[24,41], such as the entrapment of the complexes and degradation of pDNA inside endo-lysosomes, difficulties in the release of pDNA from the complex (our results showed strong electrostatic interactions between the vectors and pDNA), low pDNA diffusion inside cytoplasm, pDNA degradation in the cytoplasm, and inifficient nuclear translocation of pDNA.


2h
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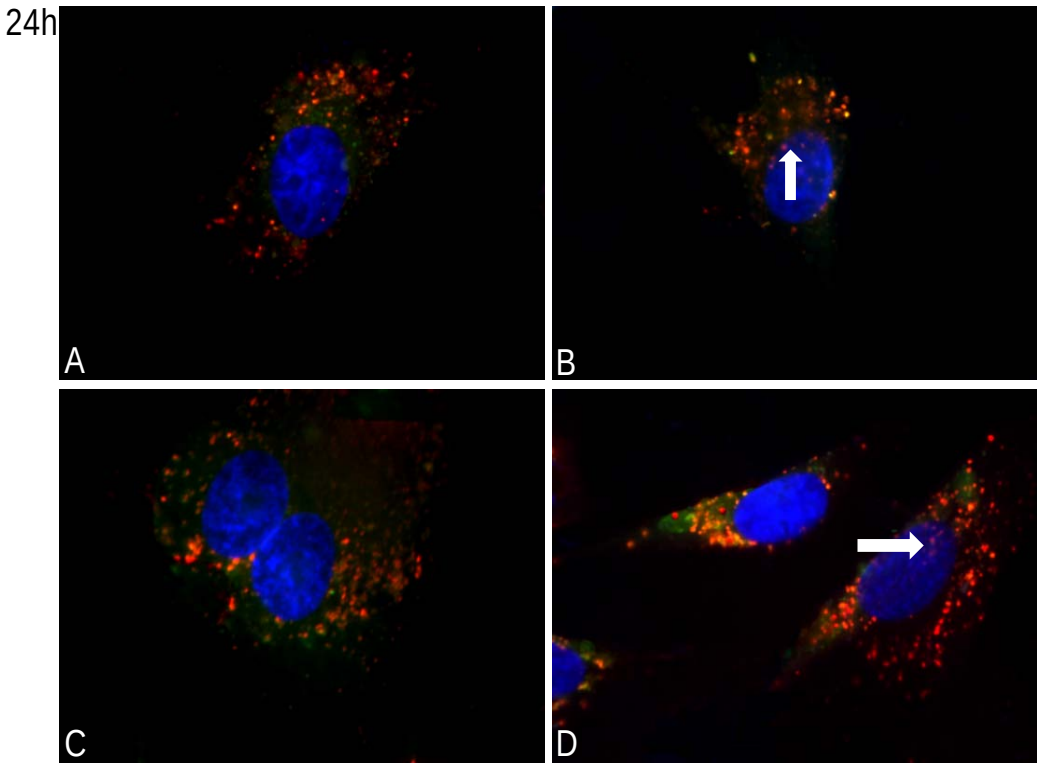

Figure 8. Cellular distribution of (RITC)-labeled pDNA in MSCs at 2 h, 4 h and 24 h post transfection. (A), (B), (C), and (D) represents cells transfected using G5, RGD8-G5, G6 and RGD8G6 vectors, respectively. The cell nuclei were stained with DAPI (blue), and the acidic late endosome and lysosome compartments were stained with LysoSensor Green DND-189 (green). The bar represents 10 μm.

3.4. Gene delivery using the PAMAM dendrimers/RGD conjugates

The ability of PAMAM dendrimers/RGD conjugates to efficiently deliver exogenous genes was evaluated using a plasmid encoding Enhanced Green Fluorescent Protein (EGFP) and Luciferase (Luc) and, after, for selected conditions, using a plasmid encoding BMP-2. Indeed, whereas EGFP and Luc are used due to their fluorescence and luminescence properties and easy detection, BMP-2 is a protein known for inducing the proliferation and differentiation of MSCs towards the osteoblastic lineage[42]. The idea was to confirm if the dendrimers/RGD conjugates were indeed able to deliver the gene encoding a protein with pharmacological relevance with an effective expression of that protein in culture.

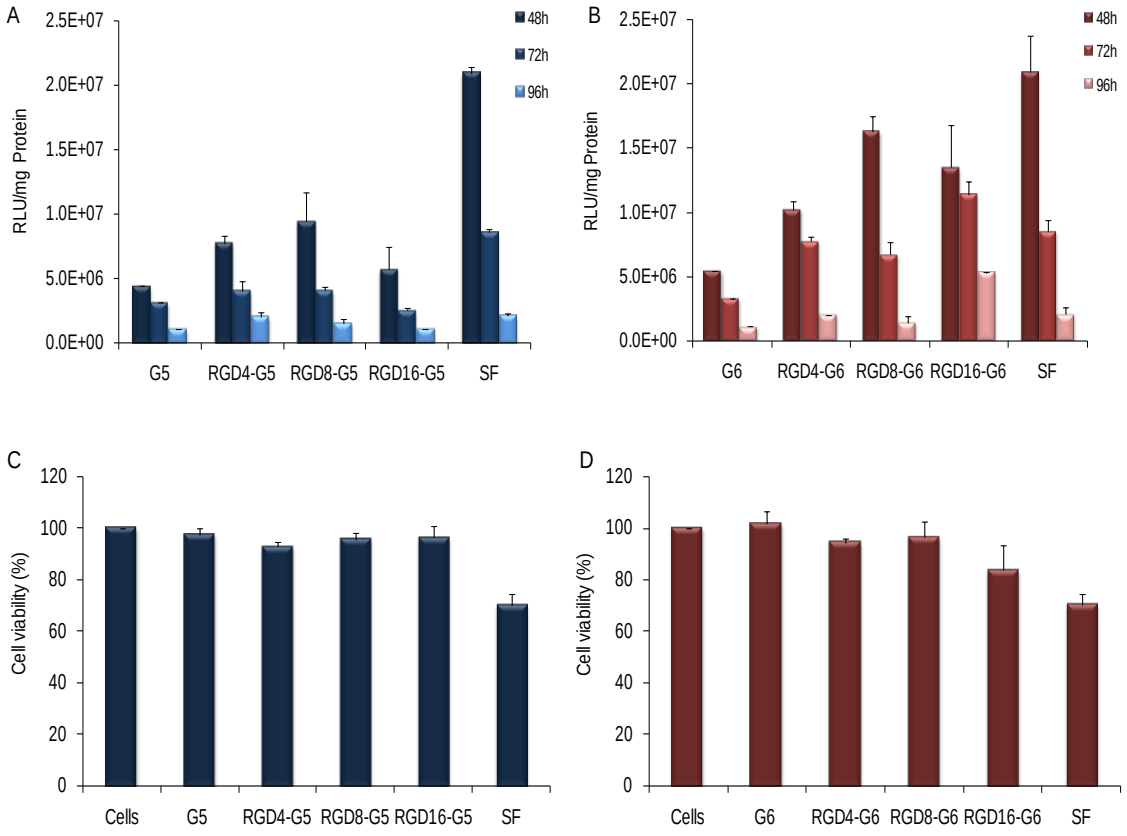

Figure 9. (A) Luc gene expression achieved with G5 dendrimer based vectors. (B) Luc gene expression achieved with G6 dendrimer based vectors. (C) Cytotoxicity evaluation of the complexes formed by pDNA and G5 dendrimer based vectors, 48 h post-transfection. (D) Cytotoxicity evaluation of the complexes formed by pDNA and G6 dendrimer based vectors, 48 h post-transfection. Results are expressed as the mean ± s.e.m., n=6.

For the first set of gene delivery studies, MSCs were incubated with the complexes in optimized conditions in the presence of 10% FBS, and for 4 h. Cells were then assayed for expression of the luciferase (Luc) reporter gene after 48, 72 and 96 h post-transfection (Figure 9). Results were normalized to protein content and are shown as relative light units (RLU). A transient expression of the Luc gene was obtained in all cases as RLU values decrease with time. After a 48 h period, dendrimer/RGD conjugates lead to higher gene delivery efficiencies than the correspondent native dendrimers ($p<0.05$). After 72 and 96 h, only G6 dendrimer based vectors presented gene expression values superior to those shown by native dendrimers. In fact, the improvement of gene expression was higher for G6 dendrimer based vectors and, in particular, for the RGD8-G6 vector. After 48 h, the RGD8-G6 vector presented a transfection level that almost reached the level obtained with SF (used as reference) and was approximately 5-fold higher than that obtained with the native G6 dendrimer. Interesting, the gene expression pattern

after 48 h was similar to that observed in the cellular uptake studies suggesting that gene expression is dependent on the amount of complexes internalized by cells that, in turn, will depend on the balance between the nanoclusters RGD density and the electrostatic interactions established between the complexes and the cell membrane.

Gene delivery achieved by dendrimer/RGD conjugates was also qualitatively studied, 24 h post-transfection, by visualization of Enhanced Green Fluorescent Protein expression using fluorescence microscopy (Figure 10). These results were in accordance with the quantitative analysis using the Luc reporter gene.

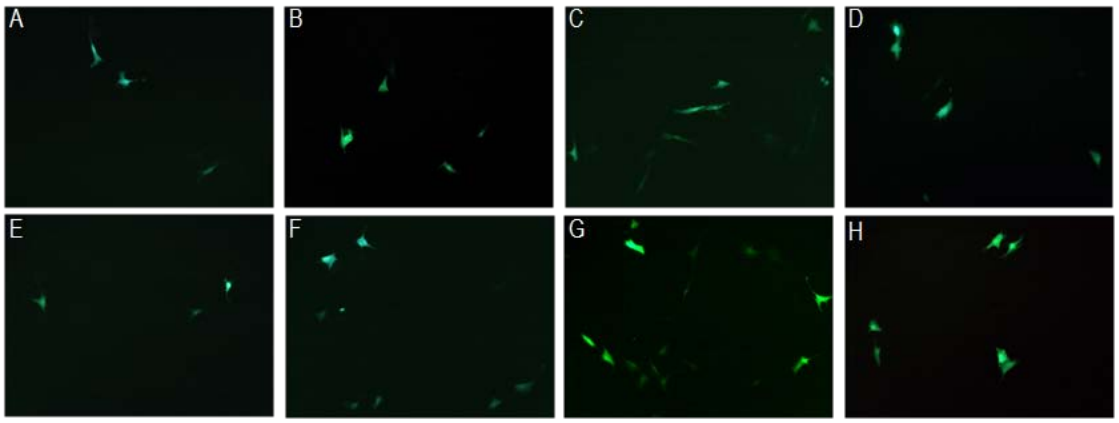

Figure 10. Fluorescence microscopy images showing Enhanced Green Fluorescent Protein expression 24 h post-transfection. The micrographs (A), (B), (C) and (D) are respectively the transfected MSCs with G5, RGD4-G5, RGD8-G5 and RGD16-G5. The micrographs (E), (F), (G) and (H) are respectively the transfected MSCs with G6, RGD4-G6, RGD8-G6 and RGD16-G6. The micrographs were obtained at an original magnification of 100×

Cell viability was evaluated simultaneously to transfection experiments using the resazurin reduction assay (Figure 9). Results are presented as the percentage of cell viability in relation to non-transfected cells. For most of the cases, cell viability was close to 100% (only the complexes formed by pDNA and the RGD16-G6 vector showed some cytotoxicity but, even so, the percentage of cell viability was above 80%). SF was more toxic than native dendrimers and dendrimer/RGD conjugates[43].

Studies on the cytotoxicity of vectors alone (not complexed with pDNA) were also performed (Figure 11). As expected, cytotoxicity was dependent on concentration but, till 25 $\mu g.mL^{-1}$ (the concentrations used in the present study were included in this interval), all the

systems displayed a high level of cell viability (>60%). It is well known that at high concentrations, the cationic charge of the dendrimers may lead to a strong cytotoxic effect[22,24].

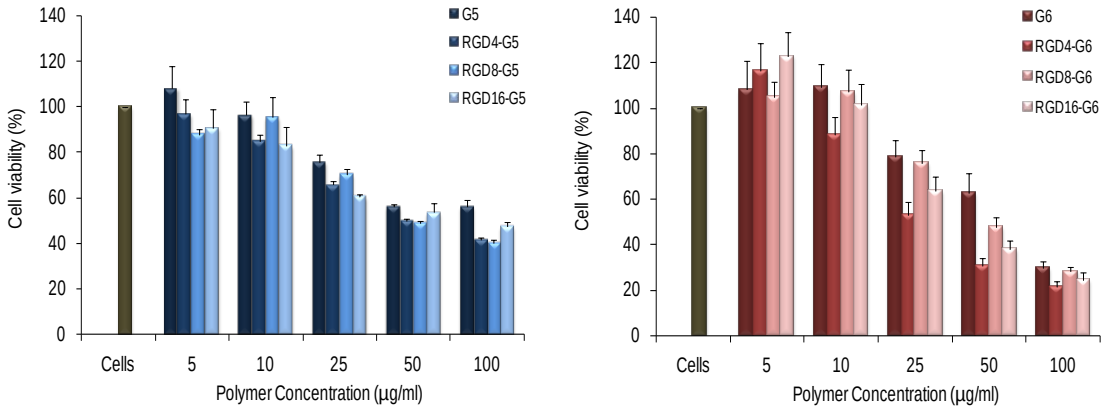

Figure 11 Cytotoxicity evaluation of dendrimers/RGD conjugates without being complexed with pDNA . Data represents the mean ± s.e.m., n=6.

The second set of gene delivery studies was carried out using a plasmid encoding BMP-2. Similar experimental conditions to those used with the EGFPLuc pDNA were applied but expression of the protein was measured after 48h post-transfection (Figure 12). The dendrimer/RGD conjugates were able to deliver the BMP-2 gene to MSCs, being in general more efficient than native dendrimers. The trend in gene delivery was identical to that observed in the first set of gene delivery assays and the RGD8-G6 vector was shown to be the more efficient, doubling the amount of protein expressed in comparison to G6 native dendrimer. Also here, together with the electrostatic interactions, RGD clustering modulated the gene delivery process and resulted in an improved gene expression of a therapeutic gene.

Several authors introduced the RGD motif in the design of gene delivery vectors with the aim of increasing gene expression[8,13-15]. In the present work, by conjugating dendrimers with a varying number of RGD containing peptides, it is shown that the electrostatic interaction established between the dendrimer moieties and the cell membrane is important and must be taken into account in the gene delivery process but, also, that gene transfer increases with nanoclusters RGD density. Results are in agreement with other authors that pointed out the importance of RGD clustering in the gene transfer process[11,13].

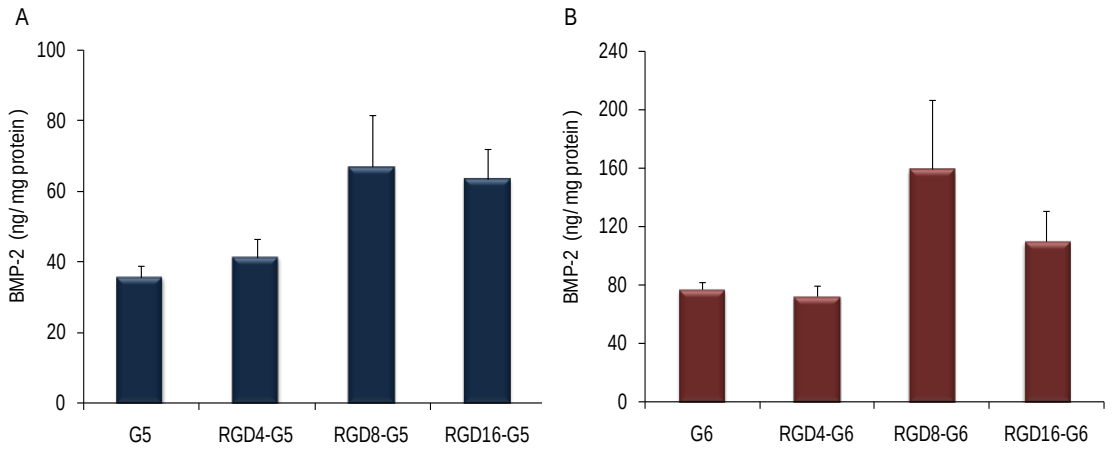

Figure 12. BMP-2 gene expression achieved with (A) G5 dendrimer based vectors and (B) G6 dendrimer based vectors. Results are expressed as the mean ± s.e.m., n=3.

In one of those works, in an attempt to mimic adenovirus entry in host cells, 5 nm gold nanoparticles were surface modified with RGD containing peptides via thiol/gold chemisorption[13]. They were then used to modify the surface of complexes formed by DNA and poly(ethyleneimine) used in gene delivery into cells with $\alpha_v\beta_3$ integrin receptors (HeLa cells). The authors observed an increase in gene delivery that was dependent on the integrin surface density of cells. Instead of gold nanoparticles, we use dendrimers that have similar dimensions (5.4 nm and 6.7 nm, respectively, for the G5 and G6 PAMAM dendrimer). In our approach, the dendrimer/RGD conjugates present the advantage of being simultaneously used as gene carriers (without the need of an additional molecule for that effect) and as scaffolds for RGD clustering.

4. CONCLUSIONS

RGD nanoclusters were formed by conjugation of PAMAM dendrimers with a varying number of peptides containing the RGD motif having in view their use as gene delivery vectors. The complexes formed by these nanoclusters and pDNA were able to enter inside MSCs through a mechanism that results from a balance between the non-specific electrostatic interactions of the complexes with the cell surface and the specific interactions established through the RGD/integrin recognition system. The density of RGD motifs in the dendrimeric scaffold was shown to play a role in complex cellular uptake mechanism and also in gene expression. The system with best performance was based on the G6 PAMAM dendrimer conjugated to eight peptide arms (RGD8-

G6). This vector presented a 2-fold higher BMP-2 expression in comparison to the G6 native dendrimer.

Due to the role of integrins in the modulation of cell processes, these dendrimeric RGD nanoclusters may have important biomedical applications, not only in gene delivery, but also in other fields such as in tissue engineering and regeneration.

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CHAPTER IV.

Functionalization of Poly(amidoamine) Dendrimers with Hydrophobic Chains for Improved Gene Delivery in Mesenchymal Stem Cells

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ABSTRACT

A new family of gene delivery vectors is synthesized consisting of a medium-size generation PAMAM dendrimer (generation 5, with amine termini) core randomly linked at the periphery to hydrophobic chains that vary in length (12 to 16 carbon alkyl chains) and number (from 4.2 to 9.7 in average). The idea subjacent to the present work is to join the advantages of the cationic nature of the dendrimer with the capacity of lipids to interact with biological membranes. Unlike other amphiphilic systems designed for the same purpose, where the hydrophobic and hydrophilic moieties coexist in opposite sides, the present vectors have a hydrophilic interior and a hydrophobic corona. The vectors are characterized in respect to their ability to neutralize, bind and compact plasmid DNA (pDNA). The complexes formed between the vectors and pDNA are analyzed concerning their size, ζ-potential, resistance to serum nucleases, capacity of being internalized by cells and transfection efficiency. These new vectors show a remarkable capacity for mediating the internalization of pDNA with minimum cytotoxicity, being this effect positively correlated with the -CH_2- content present in the hydrophobic corona. Gene expression in MSCs, a cell type with relevancy in the regenerative medicine clinical context, is also enhanced using the new vectors but, in this case, the higher efficiency is shown by the vectors containing the smallest hydrophobic chains.

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1. INTRODUCTION

Gene therapy is a branch of medicine that aims to replace deficient genes, modulate gene expression or integrate new genes (new functions) in cells[1,2]. In fact, nowadays, gene therapy is not only concerned with genetically inherited diseases, but also with the treatment of acquired diseases (by interfering with gene expression)[3,4] and with tissue engineering and regeneration[5,6]. Bone marrow derived mesenchymal stem cells, being multipotent cells with the ability to differentiate into multiple lineages, including the osteogenic, chondrogenic, myogenic and adipogenic lineages, hold tremendous promise for the construction and regeneration of tissues[7,8]. Furthermore, they are relatively easy to isolate, manipulate and culture, and, at least *in vitro*, show significant expansion capability[7,8]. These characteristics make them very interesting candidates for the delivery of exogenous genes.

A wide variety of viral systems (e.g. retroviruses, lentiviruses, adenoviruses or baculoviruses) have been extensively investigated as gene delivery vectors, including in MSCs[9]. These carriers have proved to be very efficient leading to high levels of expression of the delivered genes. However, their application in the clinical scenario as a successful approach has not yet met expectations as viruses pose serious problems in terms of safety (e.g. immune responses)[10]. Because of such inherent risks, non-viral gene delivery has been pursued as an alternative strategy. Moreover, very often, a long-term expression of the delivered gene (that can be obtained using viruses) is normally not required. That is the case of tissue engineering and regeneration approaches for which transient signaling is ideally needed[11]. Therefore, a wide range of non-viral synthetic vectors has emerged based on lipids, classical polymers, dendrimers, polypeptides and inorganic nanoparticles[12]. Despite the advantages of these systems over their viral counterparts, some of them show significant citotoxicity and all exhibit low gene delivery efficiency when dealing with primary cells. MSCs, in particular, have been shown to be very difficult to transfect by non-viral methods[13,14].

Among the numerous non-viral vectors investigated, dendrimers have great potential over the traditional polymeric systems as a result of their unique characteristics[15,16]. Dendrimers are hyperbranched polymers that present well-defined three-dimensional architectures (they are organized in layers called generations), molecular weights, and size, as well as a multivalent-

functionalized terminal surface. Poly(amidoamine) (PAMAM) dendrimers, with primary amine termini and tertiary internal amines, are protonated at physiological pH and can form complexes with the anionic deoxyribonucleic acid (DNA) molecule. PAMAM dendrimers have the intrinsic ability to bind, condense and efficiently deliver DNA into a wide variety of cell types without inducing significant cytotoxicity and immunogenicity[17-22]. It is accepted that the primary amine groups at the periphery bind and compact DNA, thus promoting its cellular uptake, while the buried tertiary amino groups act as a proton sponge in endosomes, thus enhancing the release of DNA into the cytoplasm[17-22]. Early studies conducted by Baker *et al.* showed that high transfection efficiency was achieved using generations 5 – 10 of PAMAM dendrimers, even if results varied significantly among the cell lines tested[23]. In a previous study, believing that these results could be extended to MSCs, we have used generations 5 – 7 of PAMAM dendrimers for MSCs transfection using a plasmid encoding hBMP-2 protein[24]. Although the process resulted in osteogenesis promotion, the level of gene expression attained in those *in vitro* assays was low. Parallel experiments using HEK 293T cells (a human embryonic kidney derived cell line) and the *β*-galactosidase reporter gene revealed higher levels of gene delivery. Indeed, these results confirmed that PAMAM dendrimers were able to deliver genes into cells but that the process was strongly cell type dependent.

PAMAM dendrimers have been modified in different ways having in view an improvement in their performance as nucleic acid carriers. Several reviews have been specifically devoted to this topic[17-21]. Physical modifications like those conducting to partially degraded PAMAM dendrimers have been reported – actually, these dendrimers are being sold in the market under the name of SuperFect® (SF) and PolyFect®[25] and have been shown to transfect a series of cell types. Chemical modifications are also described in the literature, the majority involving functionalization at the dendrimers periphery, such as conjugation with poly(ethyleneglycol), cyclodextrins, amino-acids, peptides, proteins, and glucocorticoids[17-21]. In particular, dendrimers having hydrophobic amino-acid residues as terminal groups were shown to have improved transfection properties[26]. Additionally, PAMAM dendrons (asymmetric partial dendrimers) differently functionalized at their focal points have been synthesized and tested for gene delivery. Despite all these studies, progress has been moderate and new strategies are needed for enhancing the gene delivery activity of PAMAM dendrimers.

Herein, we focused on the possibility of associating the advantages of the cationic nature of amine terminated PAMAM dendrimers with the capacity of lipids to interact with biological membranes. Takahashi *et al.*[27-29] synthesized low generation PAMAM dendrons (generations 1 – 4) bearing two dodecyl tails and concluded that transfection activity increased with the generation of the dendron in CV1 cells (the fusogenic lipid dioleoylphosphatidylethanolamine was added during the process). An increment in the length of the alkyl chain (octadecyl chains) positively influenced cell transfection only when serum was present showing that the hydrophobic moieties played a role in the stability of the complexes formed between the cationic lipids and DNA. Other asymmetric dendrimers with potential for gene delivery have been reported. Jones *et al.*[30] combined cholesterol units with spermine-functionalized dendrons. Using solid phase methods, Shah *et al.*[31] linked branched polypeptides to a hydrophobic tail containing 14 carbons. Ewert *et al.* and Ahmad *et al.*[32,33] synthesized cationic lipids with dendritic head groups based on the aminoacid ornithine. Joester *et al*.[34,35] prepared a series of low generation amphiphilic dendrimers through connection of a lipophilic dendron with a hydrophilic dendron by a rigid diphenylethyne core. All these systems, shared a common feature – the dendritic hydrophilic moiety is presented at one side of the vector and the hydrophobic moiety in the opposite side.

In this study, we followed a different strategy and synthesized a new family of gene delivery vectors consisting of a medium-size generation PAMAM dendrimer core randomly linked at the periphery to hydrophobic chains that varied in length and number. Generation 5 PAMAM dendrimers (G5) were chosen due to their high amine content both at the periphery and in the interior. We hypothesized that the strong positive character of the dendrimer would favor complexation with DNA, the electrostatic attraction between the complex and the negatively charged cell membrane, and the release of DNA into the cytosol due to the proton sponge effect. The hydrophobic tails would act synergistically with the cationic core, facilitating the transport of the complexes across the cell membrane and helping the escape of the complexes to the cytosol by fusing or destabilizing endosomes. Lauric (dodecanoic acid, 12 carbons, La), myristic (tetradecanoic acid, 14 carbons, My) and palmitic (hexadecanoic acid, 16 carbons, Pa) fatty acids were chosen to be conjugated to dendrimers to evaluate the effect of the length of the hydrophobic chains on the process of transfection and also because they have an endogenous nature and can be metabolized by mammalian cells. Indeed, these medium chain fatty acids have been shown to be absorption enhancers and to play a role in the intracellular trafficking of

proteins[36,37]. Low generation PAMAM dendrimers conjugated with lauroyl chloride were also shown to have enhanced permeation through Caco-2 cell monolayers[37]. In the present work, the effect of the extent of functionalization was studied by binding a different number of hydrophobic chains per dendrimer. Furthermore, MSCs were used in the biological assays instead of established cell lines, allowing a more reliable extrapolation of the results for an *in vivo* situation.

Herein, a new family of gene delivery vectors was developed, which showed a remarkable capacity for mediating the internalization of pDNA without significant cytotoxic effects. Nucleic acid cellular uptake was positively correlated with the $–CH_2–$ content present in the dendrimer hydrophobic corona. Gene expression was also greatly enhanced using the new vectors but, in this case, the higher efficiency was presented by the vectors containing the smallest hydrophobic chains.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Generation 5 PAMAM dendrimers (G5) were purchased from Dendritech Inc., USA. Plasmid DNA (pDNA) encoding for Enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) with a cytomegalovirus promoter (CMV) was kindly provided by Prof. Tatiana Segura (UCLA, USA). pDNA was purified from overnight E. Coli bacteria culture using the GenElute™ HP Endotoxin-Free Plasmid Megaprep Kit and stored in ultrapure water at -20 ºC. All other reagents used in the synthesis, if not specified, were obtained from Sigma-Aldrich Co. and used without further purification. Cell culture dishes were from Nunc.

2.2. Experimental determination of primary amine group content of dendrimers

G5 primary amine group content was determined by spectrophotometry after reaction of the free amine groups with TNBS[38], as previously described, and using glycine as standard. A total of 115 amine groups was attributed to each dendrimer, being this value in agreement with

the Mass Spectrometry data supplied by the seller. Based on this result, the TNBS method was further used to calculate the concentration of dendrimers (also of functionalized dendrimers) when needed. In both cases the standard solutions and the sample solutions were serially diluted in 0.1 M sodium tetraborate to a final volume of 1 ml. To each standard and sample, 25 μL of TNBS (0.03 M) diluted in water were added. After 15 min at room temperature (RT), absorption was measured at 420 nm in a GBC-Cintra 40, UV-Visible spectrophotometer.

2.3. Synthesis and characterization of the functionalized dendrimers

In a first step, lauric, myristic, and palmitic fatty acids were activated using carbodiimide chemistry as described by Lapidot *et. al.*[39] with slight modifications. Briefly, the fatty acids (0.025 mol) were dissolved in THF (20 ml) and purged with nitrogen. Then, 0.025 mol of N-hydroxysuccinimide (NHS) was added and the contents were stirred at 0 ºC. Activation was initiated by dropwise addition of dicyclohexylcarbodiimide (DCC, 0.025 mol in 20 ml THF) into the above mixture to obtain a reaction ratio of 1:1:1. This mixture was further stirred at RT and under nitrogen for 12 h. Dicyclohexylurea, a by-product, was removed by filtration and the filtrate was concentrated under reduced pressure to yield white crystals and purified by recrystallization in methanol (a 90 % yield was obtained). In a second step, the functionalized dendrimers were synthesized by N-acylation reaction of G5 with the activated ester of the fatty acids (Figure 1). The degree of functionalization was controlled using the stoichiometry of the reagents. The adequate amount of activated fatty acid was dissolved in 1 ml of DMF and added dropwise over 15 min to 30 mg of G5 dissolved in 1 ml of DMSO. The mixture was allowed to stir 16 h under nitrogen and at RT. The reaction mixture was concentrated under reduced pressure and purified by extensive dialysis against Phosphate Buffered Saline (PBS) solution and ultra-pure water for 4 days. All the products presented good water solubility. The conjugates were sterile filtered and aliquots were frozen at -20 ºC. The conjugates were characterized by ^{1}H NMR. Spectra were recorded at RT, using a Bruker 400MHz Avance II^{+} NMR spectrometer using D_2O as solvent. The G5 content of each sample was determined by the TNBS assay. The integrals of the characteristic proton peaks of the fatty acids ($\delta \approx 0.8$, CH_3) and of G5 ($\delta \approx 2.3$, CH_2) were used to calculate the average number of hydrophobic chains per dendrimer.

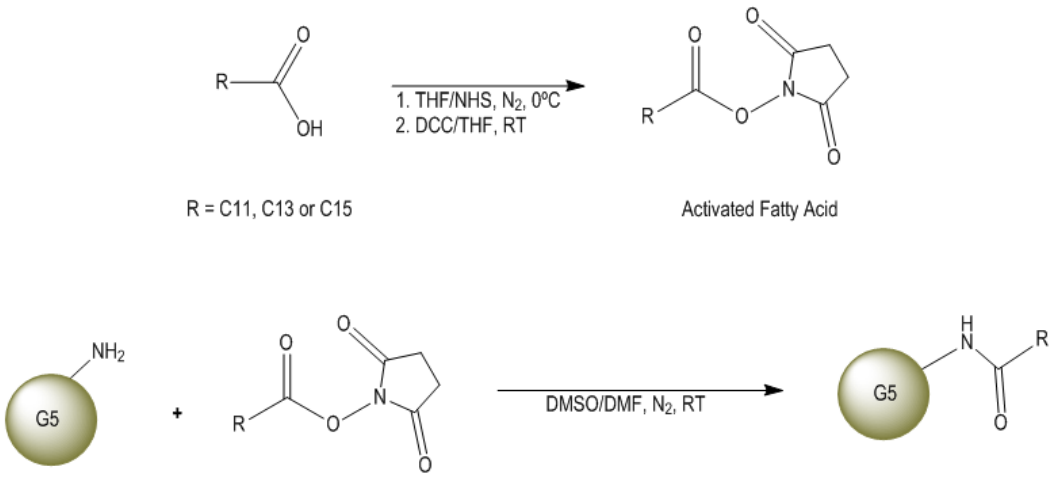

Figure 1. Strategy of synthesis followed in dendrimer surface functionalization with alkyl chains (activation of the fatty acid + conjugation reaction)

2.4. Functionalized dendrimers/pDNA complexes assembly

Functionalized dendrimers/pDNA complexes were prepared at several N:P ratios by mixing equal volumes (50 µL) of the conjugate solution and the pDNA solution. Unless otherwise stated, Hepes-Buffered Glucose (HBG, Hepes 10 mM, Glucose 5% w/w, pH = 7.1) was always used to prepare these solutions. Complex solutions were gently vortexed and allowed to incubate for 20 min at RT prior to experiments. Unmodified G5 was always used as a control. Superfect® (Qiagen, Germany) was also used as a reference in gene delivery experiments. SF complexes were prepared accordingly the manufacturer's instructions for transfection of primary cells.

2.5. Agarose gel electrophoresis shift assay

Agarose gel (0.7% w/v) containing ethidium bromide (0.05 µL.ml^{-1}) was prepared in Tris-acetate-EDTA buffer. Complex solutions were prepared at different N:P ratios as described above using 1 µg of pDNA diluted in 50 µL of buffer and 2.5 µL of Blue Loading Buffer (Fermentas, Germany) were added before the samples were subjected to gel electrophoresis at 75 V. Functionalized dendrimer-pDNA binding was shown by a lack of migration of the pDNA in the electrophoretic field.

2.6. Pico green intercalation assay

200 µL of complex solutions at different N:P ratios were prepared as described above using 0.1 µg of pDNA diluted in 100 µL. Hepes-buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.0) was used to prepare these solutions. Then, 200 µL of PicoGreen (PG, Molecular Probes) reagent diluted in Tris-EDTA buffer (TE, 10 mM Tris, 1 mM EDTA, pH 7.5) was added and mixtures were further incubated for 5 min. Three independent experiments were performed. PG fluorescence (λ_{ex} = 485 nm, λ_{em} = 535 nm) was measured using a microplate reader (model Victor^3 1420, PerkinElmer). The % relative fluorescence (%F) was determined using the following equation:

$$\%F = 100 \times \frac{F_{sample} - F_{blank}}{F_{DNAonly} - F_{blank}}$$

2.7. Serum nucleases protection assay

The procedure followed was modified from a method described by Gao *et al*.[40]. In brief, functionalized dendrimers (5 µL) were assembled with 1 µg of pDNA (5 µL) as described above, at an N:P ratio of 5. The complexes were then incubated with 5 µL of fetal bovine serum (FBS, Gibco), at 37ºC, for 0, 1, and 4 h. At the end of the incubation period, 5 µL of sodium dodecyl sulphate (SDS, 4% w/v) was added to release the pDNA from the functionalized dendrimers, and the samples were incubated overnight at 60 ºC to facilitate the release of pDNA. Blue Loading Buffer (4 µL) was then added to the samples and 10 µL of each was loaded onto the gel (0.7% w/v agarose) and subjected to electrophoresis. pDNA and FBS were used as negative controls.

2.8. Dynamic light scattering and zeta potential measurements

The size of complexes was measured at 633 nm on a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments). Solutions (100 µL) of polyplexes were prepared at a N:P ratio of 5 as described above using 5 µg of pDNA diluted in 50 µL. The solutions were then

diluted by adding 700 µL of HBG buffer. Particle sizes were determined for these initial solutions at room temperature with a detection angle of 173°. Zeta potential measurements were performed using the same instrument with a detection angle of 17°. Zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions. The data presented are means of three independent sample measurements.

2.9. Isolation and culture of rat bone marrow-derived mesenchymal stem cells

Rat bone marrow-derived mesenchymal stem cells (MSCs) were isolated from long bones of 8-week-old male Wistar rats according to a standard protocol[41]. Following euthanasia by pentobarbital 20% (v/v), femora were aseptically excised, cleaned of soft tissue, and washed in PBS. The metaphyseal ends were cut off and the marrow was flushed out from the midshaft with 5 ml of α-Minimum Essential Medium (α-MEM) using a 23-Gauge needle and syringe. The cells were centrifuged (600 g, 5 min), suspended in fresh medium containing 10% heat-inactivated FBS, 50 $mg.l^{-1}$ ascorbic acid, 100 $U.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ of streptomycin - the basic medium - and seeded in 75 cm^2 flasks. After removal of non-adherent cells and medium exchange at day 3, cells were harvested at day 7, and used in the experiments.

2.10. Cellular uptake studies using fluorescence-activated cell sorting (FACS)

Cells were seeded in twelve-well plates at a density of 1.25×10^4 $cell.cm^{-2}$ and incubated in basic medium at 37ºC, 5% CO_2, for 24 h, to yield a cell confluency of around 60-70%. Prior to complex formation, pDNA was labelled with PicoGreen dye (Molecular Probes) according to the manufacturer's instructions. Complex solutions (100 µl) were prepared at a N:P ratio of 6 as described above. Cells in 0.5 ml of basic medium were then transfected using 100 µl of the complex solution (2 $\mu g.cm^{-2}$ of pDNA was used). The cells were incubated with each solution for 1 h and then rinsed twice with PBS. The extracellular fluorescence associated with cell surface-bound nanoparticles was quenched with 0.4% (w/v) TB for 5 min. The cells were trypsinized, pelleted, and resuspended in 400 µl PBS containing 2% FBS for fluorescence-activated cell sorting (FACS) analysis (Cytomics FC500, Beckman Coulter). Twenty thousand events were

collected in triplicate for each sample. Gating and analysis was performed using CXP software analysis program using PG-labelled naked pDNA transfected cells as the primary negative control. The positive fluorescence level was established by visual inspection of the histogram of the negative control such that less than 1% of positive cells appeared in the positive region. Results are expressed as the percentage of fluorescent cells and the fluorescence intensity per cell (the mean value). Three independent experiments were performed.

2.11. Gene delivery studies- expression of the Luc gene

Two independent experiments were performed and all samples were carried out in triplicate. Cells were seeded at 1.25×10^4 cell.cm^{-2}, in 24-well plates, 24 h prior to transfection. At the time of transfection, cells reached a confluency of 60-70%. Before contact with complexes, medium was exchanged for 0.5 ml of fresh basic medium. Complex solutions (100 µl) were then added to the cells and, after 4 h, the culture medium was again replaced with fresh medium. Transfection was carried out using 2 µg.cm^{-2} pDNA and at a N:P ratio of 6 (optimized conditions). Non-transfected cells and cells transfected with naked pDNA were used as negative controls. At different time points (48, 72 and 120 h) after transfection began, the media was removed and the cells were washed with PBS solution and treated with 100 µl reporter lysis buffer (Promega). Cell lysates were analysed for luciferase activity with Promega's luciferase assay reagent in triplicate (following the supplier's instructions). For each sample, the microplate reader (model Victor^3 1420, PerkinElmer) was set for 3 s delay with signal integration for 10 s. The amount of protein in cell lysates was determined using the bicinchoninic acid assay (BCA assay) with bovine serum albumin as a standard. The gene delivery efficiency of each sample was characterized by firefly luciferase expression and denoted as relative light units per milligram of total protein (RLU.mg^{-1}).

2.12. Gene delivery studies - expression of the EGFP gene

Enhanced Green fluorescent protein expression studies were carried out as mentioned above for the Luc gene expression. Twenty-four hours after transfection, cells were observed with an inverted fluorescence microscope (Nikon Eclipse TE 2000E) equipped with a cold Nikon

camera. Digital image recording and image analysis were performed with the NIS Elements Advanced Research (version 2.31) software.

2.13. Cytotoxicity of the functionalized dendrimers

Cytotoxicity was evaluated by determining the percentage of cell viability (in respect to unexposed cells) using the rezasurin reduction assay that establishes a correlation between the cellular metabolic activity and the number of viable cells in culture[42]. Cell viability was studied as a function of the functionalized dendrimer type and the concentration. Cells were seeded in 96 well-FluoroNunc plates at a density of 3×10^4 cell.cm^{-2}. After 24 h, medium was replaced with fresh basic medium and 10 µl of each functionalized dendrimer (diluted in HBG) was added to achieve the final desired concentration. After 4 h, the medium was exchanged for fresh medium containing 0.1 mg.ml^{-1} rezasurin and incubated for another 4 h. Resorufin fluorescence (λ_{ex} = 530 nm, λ_{em} = 590 nm) was measured in a microplate reader (model Victor^3 1420, PerkinElmer).

Cytotoxicity of the complexes: Cytotoxicity was also evaluated using the rezasurin reduction assay. This information was obtained during the experiments performed to study the expression of the Luc gene (described above), being cell viability evaluated 24 h post-transfection.

2.14. Fluorescence microscopy studies

Fluorescence microscopy was used to visualize pDNA cellular uptake. Prior to complex assembly, pDNA was labelled with rhodamine isothiocyanate (RITC) by a slight modification of a reported method[43]. Briefly, 0.1 mg of pDNA diluted in 297 µl of sodium carbonate-buffered solution (0.1 M, pH 9.0) was mixed with 3 µl of RITC solution (100 mM solution prepared in dimethyl sulfoxide) at RT, for 5 h. The RITC-labelled pDNA was separated from residual RITC by gel filtration using a PD 10 column (GE Healthcare), followed by ethanol precipitation to obtain the RITC-labelled pDNA. Experiments were done in the same conditions as the above described transfection experiments although using RITC-labelled pDNA at a lower concentration (1 µg.cm^{-2}) to avoid blur. Live cells were analyzed under the fluorescence microscope (Nikon Eclipse TE

2000E inverted microscope) four hours post transfection after quenching of extracellular fluorescence with 0.4% TB for 5 min and washing with PBS. For observation at higher magnifications, cells were seeded in 24-well plates containing collagen-treated cover slips. Images were taken 2, 4, and 24 h post-transfection. After quenching with 0.4% TB, cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde prepared in PBS at room temperature for 10 min. Cell nuclei were then stained using a 300 nM 4',6-diamidino-2-phenylindole (DAPI) solution for another 10 min. Cells were washed several times with PBS and stored at 4ºC (protected from light). A 100x NA 0.5-1.3 Plan Fluor objective (NIKON) was used for these observations.

2.15. Statistics

Statistical analyses were performed using GraphPad Prism 5.0 for Windows. Results are reported as mean± standard error of mean (s.e.m). Unpaired, Student's t-test and 2-way ANOVA with Bonferroni Post Hoc test were used to assess the statistical differences between the group means.

3. RESULTS AND DISCUSSION

3.1. Synthesis and characterization of the functionalized dendrimers

Hydrophobic chains were conjugated to the surface of PAMAM dendrimers by N-acylation of the dendrimers with the N-hydroxysuccinimide esters of lauric, myristic, and palmitic fatty acids (Figure 1). These activated fatty acids were previously prepared using carbodiimide chemistry. The objective was to prepare a new family of gene delivery vectors consisting of a G5 core linked to 4 and 8 hydrophobic tails of different length (respectively, La1-G5, My1-G5, Pa1-G5 and La2-G5, My2-G5, Pa2-G5). During the synthesis, the concentration of dendrimers (also of functionalized dendrimers) was determined by spectrophotometry based on primary amine content, using TNBS method[38]. The conjugates were characterized by ^{1}H NMR (400MHz) using D_2O as solvent (Figure 2). Data confirm that the hydrophobic chains were successfully grafted at

the periphery of G5 as evidenced by the appearance of the characteristic proton peaks of the hydrophobic moieties: methyl protons peak (t, -CH_3) at $\delta \approx 0.8$ and methylene protons peak (m, -CH_2-) at $\Delta\delta$ = 1-1.25. Additionally, new peaks and peak splitting were observed by comparison with the G5 spectrum as a consequence of the new chemical environment. Integrals of the characteristic proton peaks of the hydrophobic moieties ($\delta \approx 0.8$, CH_3) and of G5 ($\delta \approx 2.3$, CH_2) were used to calculate the average number of hydrophobic chains effectively attached per dendrimer (Table 1). A degree of functionalization varying between 4.2 - 5.3 and of 7.8 – 9.7 was obtained for the family of gene delivery vectors prepared.

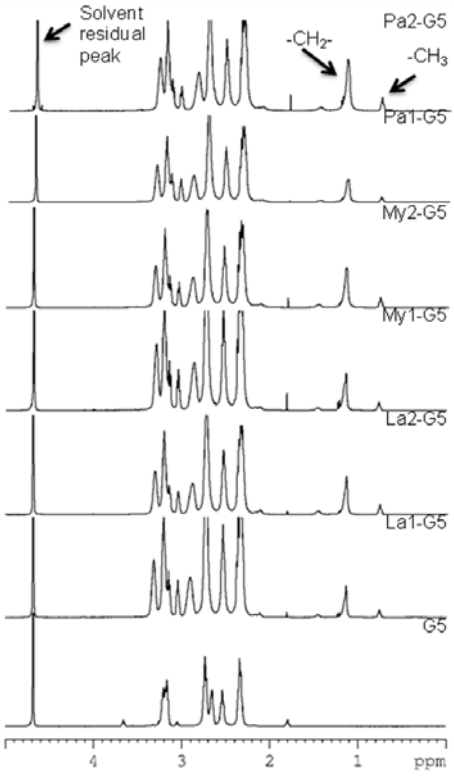

Figure 2. ^{1}H NMR (400MHz) spectra of functionalized dendrimers in D_2O.

3.2. Characterization of the complexes formed by the functionalized dendrimers and pDNA

The potential of functionalized dendrimers to neutralize, bind and compact plasmid DNA (pDNA) was studied due to its importance for efficient gene delivery. Plasmid DNA encoding for the enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) was used. Prior to all studies, conjugates were mixed with pDNA at several N:P ratios in HBG, for

complexes formation. Agarose gel shift assays (Figure 3) revealed that binding to and charge neutralization of pDNA occurred at N:P ratios of one and higher in all cases studied, except when La2-G5 was used for which a minimum N:P ratio of two was needed.

Table 1. Identification of the developed gene delivery vectors and characterization of their hydrophobic moieties.

Fatty acid used in the conjugation reaction	Vector abbreviation	Mean number of $–CH_3–$ protons per dendrimer[a]	Mean number of $–CH_2–$ protons per dendrimer[b]	Mean number of alkyl chains per dendrimer	Molecular weight, MW (u.m.a.)[c]
$CH_3(CH_2)_{10}COOH$	La1-G5	12.6	72.3	4.2	27914.4
Lauric acid	La2-G5	23.4	130.4	7.8	28569.6
$CH_3(CH_2)_{12}COOH$	My1-G5	14.0	93.9	4.7	28137.0
Myristic acid	My2-G5	27.9	177.6	9.3	29103.0
$CH_3(CH_2)_{14}COOH$	Pa1-G5	15.8	124.5	5.3	28411.4
Palmitic acid	Pa2-G5	28.9	219.0	9.7	29458.6

[a]Values used to calculate the mean number of alkyl chains per dendrimer. [b]These values refer to the $–CH_2–$ content present in dendrimer hydrophobic corona. [c]Calculated based on G5 MW provided by the supplier and the number of alkyl chains per dendrimer determined by ^{1}H NMR.

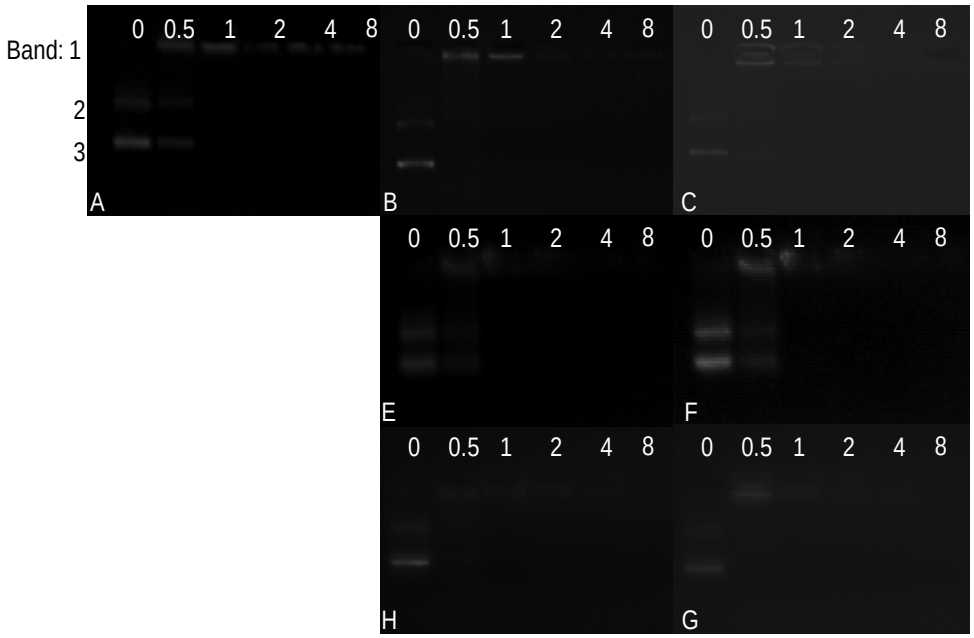

Figure 3. Agarose gel electrophoresis shift assay results for N:P ratios ranging from 0 (pDNA only) to 8: (A) G5, (B) La1-G5, (C) La2-G5, (D) My-G5, (E) My2-G5, (F) Pa1-G5, and (G) Pa2-G5. Binding is shown by the inhibition of pDNA electrophoretic mobility (band 1). Bands 2 and 3 show the relaxed and supercoiled forms of pDNA, respectively.

By using the PicoGreen (PG) dye (Figure 4), which only presents fluorescence when bounded to DNA, we concluded that complete pDNA packaging was achieved at a N:P ratio of two both for G5 and the dendrimers linked to hydrophobic chains. Based on these results, a N:P ratio of at least 5 was selected for further experiments.

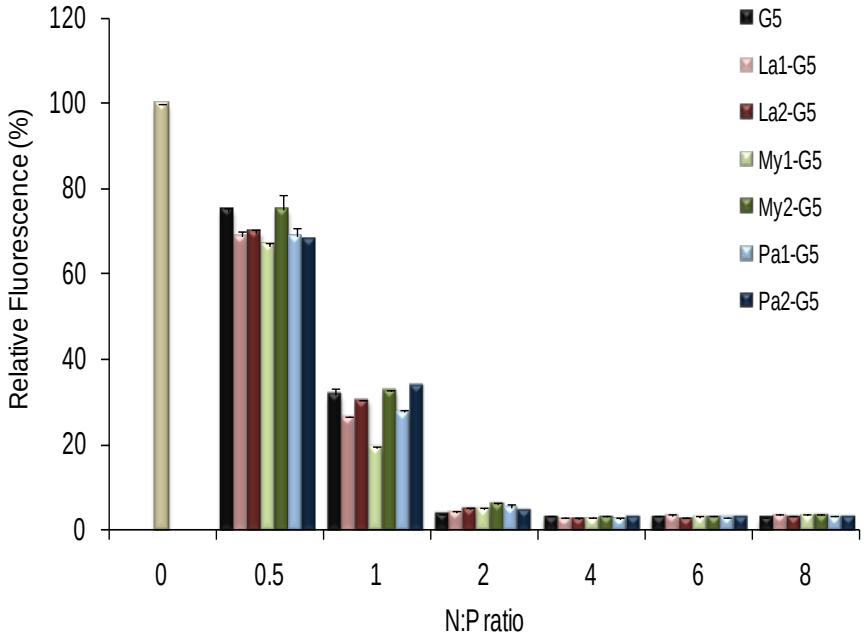

Figure 4. PicoGreen assay. The results are reported as the relative percentage of PG fluorescence, where 100% intensity was observed for a N:P of 0 (pDNA only). Results are expressed as the mean ± s.e.m obtained from three independent experiments.

At N:P=5, dynamic light scattering (DLS) and ζ-potential measurements were carried out to determine the size and colloidal stability of the formed complexes (Figure 5A and 5B). Conjugation of hydrophobic chains to G5 resulted in a significant increase ($p<0.05$) in complex mean diameter which ranged between 115 and 180 nm. In general, the size of the complexes increased with the length of the hydrophobic chain (this is particularly noticeable for the series of complexes formed by the La1-G5, My1-G5, Pa1-G5 vectors). The polydispersity indices (PDI) varied among the complexes showing that the heterogeneity of those formed by La1-G5, La2-G5, and My2-G5 vectors was lower. As expected, due to a shielding effect of the hydrophobic tails over primary amines, functionalization also gave rise to a significant decrease (20-42%, $p<0.05$) in ζ-potential values. In this case, ζ-potential values decreased with the length of the hydrophobic chain. These lower ζ-potential values associated with added hydrophobicity conferred by the fatty

acid chains, although diminishing the stability of the colloid solution (theoretically, the propensity for colloids aggregation increases), can be advantageous as interactions with charged serum constituents and, concomitantly, blood clearance can be weaken – a key issue in the *in vivo* situation[44].

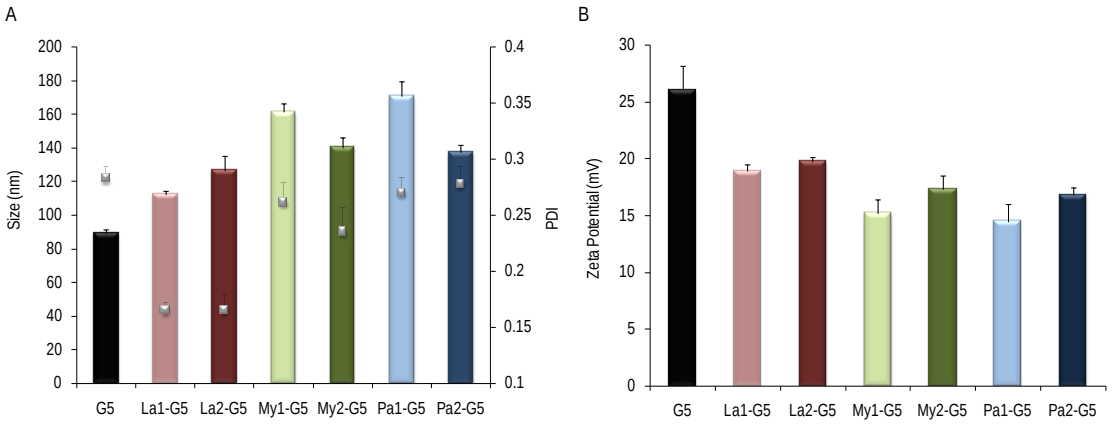

Figure 5. (A) Complex size (mean diameter) and polydispersity indices assessed by DLS, and (B) complexes ζ-potential. Results were obtained for a N:P ratio of 5. Results are expressed as the mean ± s.e.m obtained from three independent experiments.

It is known that unprotected pDNA can be rapidly degraded by nucleases present in serum, normal plasma or cytoplasm, reducing any chances of successful transfection. The complexation of pDNA with the synthetic vector, resulting in the formation of tightly packed nanoparticles, can however confer protection against nucleases action[44]. The *in vitro* resistance of the complexes formed between pDNA and the functionalized dendrimers to degradation by nucleases present in serum was also evaluated. Complexes were incubated with foetal bovine serum (FBS), at 37ºC, for 0, 1 and 4 h and, after, sodium dodecyl sulphate was added to dissociate the complexes and samples were subjected to electrophoresis (Figure 6). Degradation of naked pDNA began immediately upon addition of serum, as observed by the decrease in intensity of bands 2 and 3 (relaxed and supercoiled pDNA, respectively) as well as the migration of degraded pDNA throughout the gel. After 1 h, naked pDNA was completely degraded by nucleases present in serum. On the contrary, pDNA complexed with G5 or with the functionalized dendrimers did not shown any evidence of degradation even after incubating with serum for 4 h, at 37ºC. It should be noted that band 4 results from the combination of serum with SDS as shown in the control FBS lanes (1-3). Data revealed that the capability to protect pDNA from nucleases degradation shown

by G5 was not changed by the incorporation of the hydrophobic chains, irrespective of their length and number.

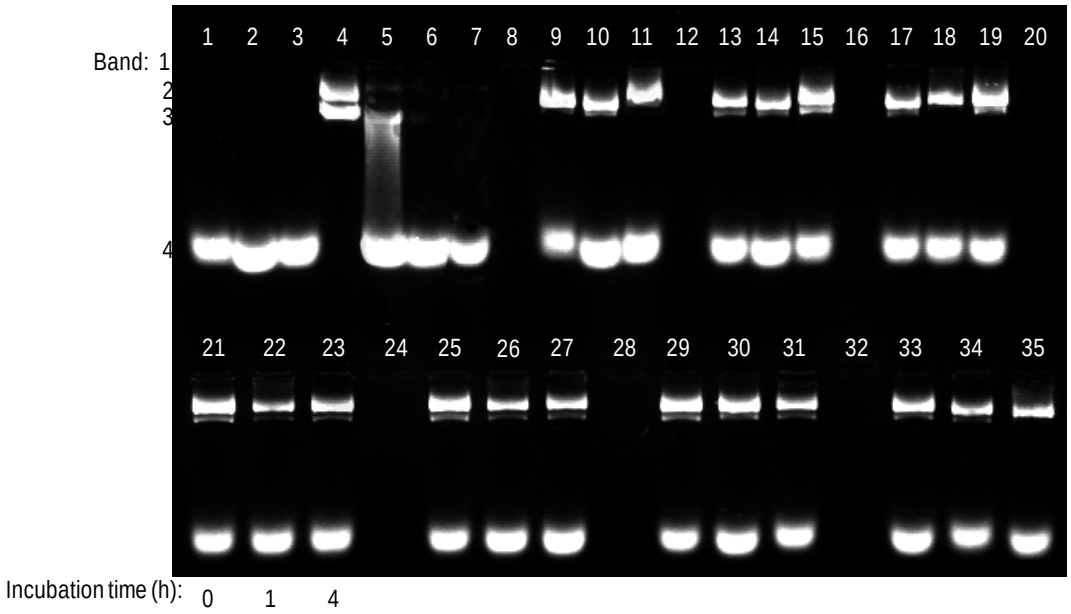

Figure 6. pDNA protection from serum nucleases achieved with G5 (lanes 9-11), La1-G5 (lanes 13-15), La2-G5 (lanes 17-19), My1-G5 (lanes 21-23), My2-G5 (lanes 25-27), Pa1-G5 (lanes 29-31), and Pa2-G5 (lanes (33-35). Results were obtained for a N:P ratio of 5. Serum only (lanes 1-3) and naked pDNA added of serum (lanes 5-7) were used as controls. Control samples without addition of serum and SDS correspondent to pDNA (lane 4) and complexes formed with G5 (lane 8), La1-G5 (lane 12), La2-G5 (lane 16), My1-G5 (lane 20), My2-G5 (lane 24), Pa1-G5 (lane 28) and Pa2-G5 (lane 32) were also analyzed. Band 1 is the position of sample loading (complexes without serum and SDS). Bands 2 and 3 are relaxed and supercoiled pDNA, respectively. Band 4 results from the complex between serum and SDS.

3.3. Cellular uptake of the complexes formed by the functionalized dendrimers and pDNA

PicoGreen-labeled pDNA was used to compare complexes uptake by rat bone marrow derived MSCs through the fluorescence-activated cell sorting (FACS) technique. Cellular uptake was studied in the presence of serum, at a N:P ratio of 5 and using a seeding density of 1.25×10^4 cell.cm^{-2} and 2 μg.cm^{-2} of pDNA. After 1h of contact between complexes and cells, both the number of cells positive for PG-labeled pDNA and the amount of pDNA delivered per cell attained with functionalized dendrimers were significantly higher ($p<0.01$ for La1-G5 and My1-G5, $p<0.05$ for La2-G5, My2-G5, Pa1- and Pa2-G5) than those obtained with G5 (Figure 7A). These values

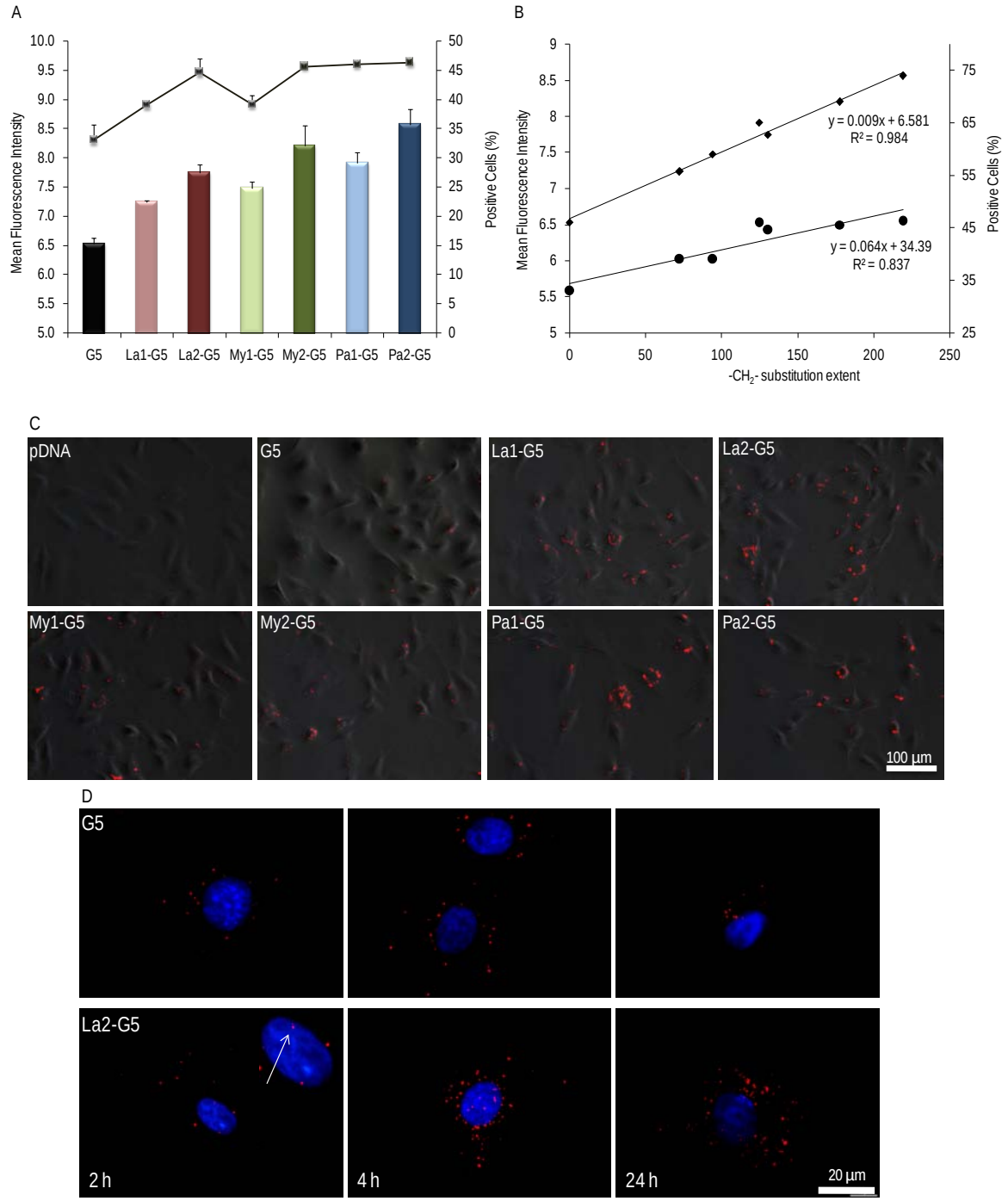

Figure 7. (A) Complex uptake by MSCs after 1h of contact. The line shows the number of cells positive for PG-labeled pDNA whereas the bars reveal the amount of pDNA delivered per cell (displayed as the mean average fluorescence intensity). Results are expressed as the mean ± s.e.m obtained from three independent experiments. (B) Correlation between the total number of –CH_2– groups contained in dendrimer hydrophobic corona and both the number of cells positive for PG-labeled pDNA (squares) and the amount of pDNA delivered per cell (circles). (C) RITC-labeled pDNA visualization in live cell cultures by fluorescence microscopy, 4 h post-transfection. Original magnifiation: 200x. (D) Distribution of RITC-labeled pDNA inside cells, 2, 4 and 24 h after transfection, when using G5 and La2-G5. Cell nuclei were stained with DAPI (blue). Original magnification: 1000x.

generally increased with the number of hydrophobic chains grafted per dendrimer (La1-G5<La2-G5; My1-G5<My2-G5; Pa1-G5<Pa2-G5 and Pa1-G5≈Pa2-G5 for, respectively, the number of cells positive for PG-labeled pDNA and the amount of pDNA delivered per cell). In addition, a linear correlation was also found between them and the total number of -CH_2- groups present in the hydrophobic chains attatched to the dendrimer (Figure 7B). It is evident that functionalization with lipids has a beneficial effect in the internalization process, probably by improving the transport of the complexes pDNA/functionalized dendrimers across the plasma membrane. Abbasi *et al.* have also found an increase in cellular uptake with increasing lipophilic character of lipid-substituted poly(L-Lysine) nucleic acid carriers[45]. The above reported results were corroborated by fluorescence microscopy images. Figure 7C shows the merged bright field and fluorescent microscopy images of MSCs 4 hours after transfection with RITC-labeled pDNA. Although these experiments provide only qualitative data, it can be seen that naked pDNA was unable to enter cells, G5 complexes were able to enter a few cells in a very small amount and functionalized dendrimer complexes entered many cells in a much higher quantity. Images taken at a higher magnification and after staining the cell nucleus with DAPI (Figure 7D), show a much higher accumulation of RITC-labeled pDNA inside cells for transfection carried out with La2-G5 (the example shown) when compared to G5. 24 hours post-transfection, pDNA is mainly located at the cell nucleus periphery although some small pink spots (due to RITC-labeled pDNA and DAPI co-localization) can be seen inside the nucleus.

Though dendrimers are reported to be internalized by cells via nonspecific adsorptive endocytosis[17-21] mainly due to nonspecific charge interactions, the present results clearly show that the hydrophobic moieties play a specific role in the process of cellular uptake. Indeed, the amount of pDNA internalized is higher when functionalized dendrimers are used, even if the charge (ζ-potential) presented by their complexes with pDNA is lower than that obtained using the native dendrimer G5.

3.4. Gene expression studies

The preceding section demonstrated the enhanced capability of functionalized dendrimers to internalize pDNA but, for protein expression, endossomal disruption, complex dissociation and

nuclear translocation of pDNA must occur[17-21]. To assess the ability of functionalized dendrimers to efficiently deliver exogenous genes, MSCs were incubated in optimized conditions in the presence of 10 %FBS, and for 4 h. Cells were then assayed for expression of the Luciferase (Luc) reporter gene after 48, 72 and 120 h post-transfection (Figure 8A). Results were normalized to protein content and are shown as RLU.

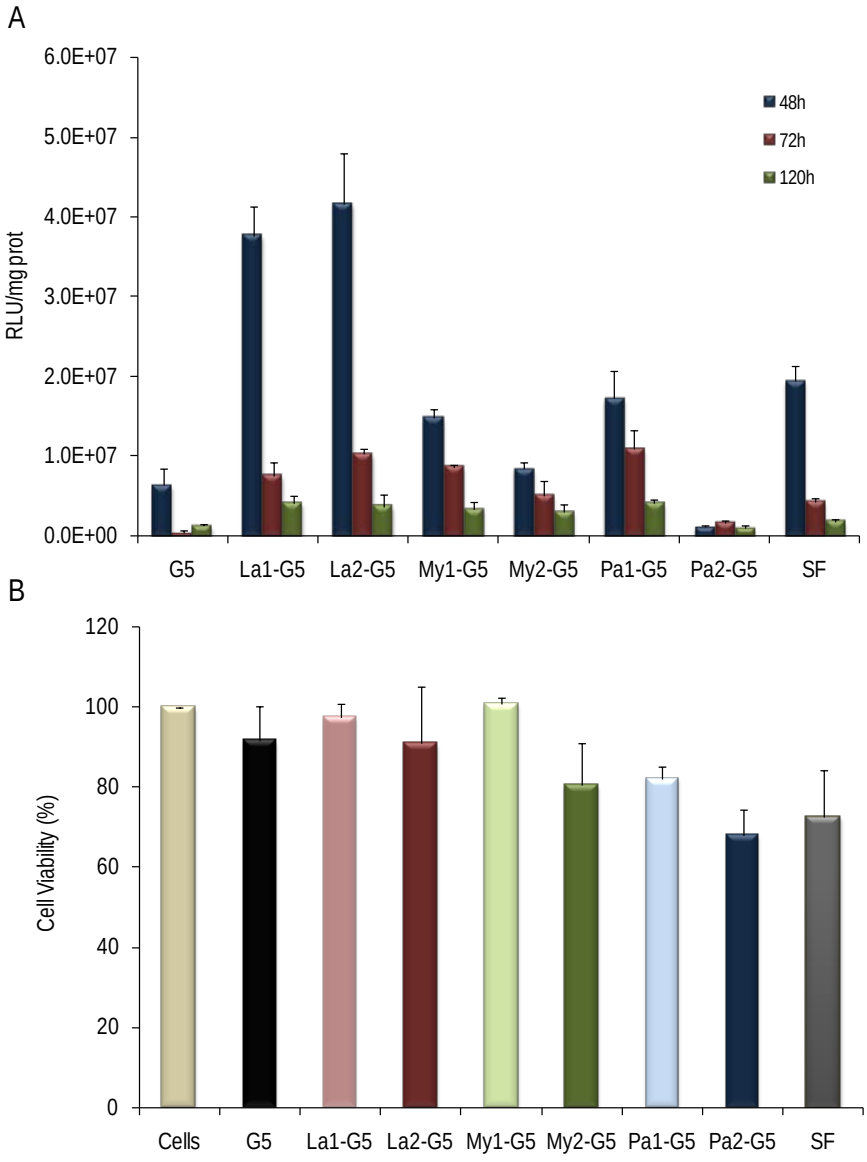

Figure 8. (A) Luc gene expression achieved with the functionalized dendrimers, 48, 72 and 120 h post-transfection. SF was used for comparison. (B) Complex cytotoxicity evaluation 24 h post-transfection. Results are expressed as the mean ± s.e.m. and were obtained from two independent experiments.

A transient expression of the Luc gene was obtained in all cases as RLU values decrease with time. For all time periods, functionalized dendrimers lead to higher gene delivery efficiencies than

G5 ($p<0.05$), with exception for the Pa2-G5 carrier. After 48h, La1-G5 and La2-G5 functionalized dendrimers presented transfection levels that were, respectively, 4.5 and 2.1-fold higher than those obtained with native G5 and SF. My1-G5 (1.8-fold higher) and Pa1-G5 (2.1-fold higher) vectors showed a moderate increase in transfection levels when compared to G5. Gene delivery efficiency mediated by the functionalized dendrimers followed the trend La1-G5≈La2-G5>Pa1-G5≈My1-G5>My2-G5>Pa2-G5, generally showing a decrease with the extent of $–CH_2–$ substitution. Results show that for a high $–CH_2–$ content, transfection efficiency can decrease to values smaller that those obtained with the native dendrimer, as happens with Pa2-G5.

Gene delivery achieved by functionalized dendrimers was also qualitatively studied, 24 h post-transfection, by visualization of Enhanced Green Fluorescent Protein expression using fluorescence microscopy (Figure 9). These results confirmed the quantitative analysis using the Luc reporter gene. Surprisingly, our findings with this new family of gene delivery vectors were different from those obtained by Takahashi *et al*.[27-29] that prepared PAMAM dendron-bearing lipids (previously mentioned). Their results showed that, in the presence of serum, the systems containing longer lipids presented higher transfection activity. Also in opposition to our results, the work dealing with lipid-substituted poly(L-Lysine)[45] concluded that the extent of $–CH_2–$ substitution positively contributed for effective DNA delivery.

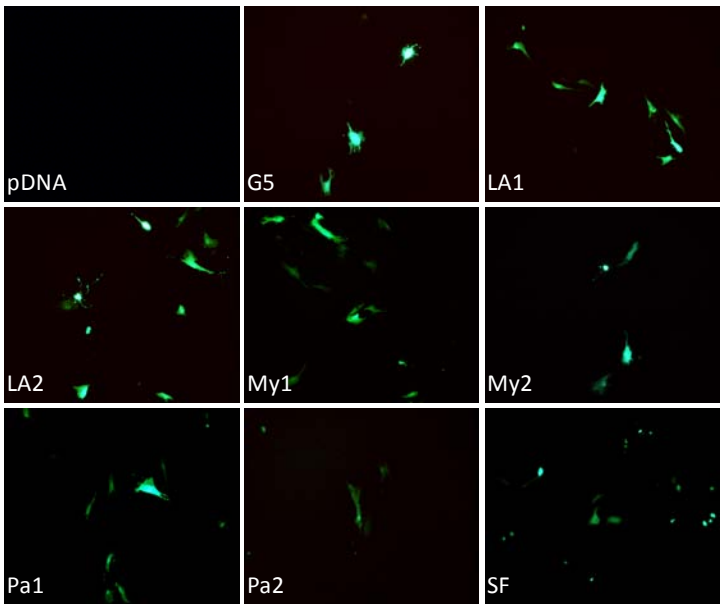

Figure 9. Fluorescence microscopy images showing Enhanced Green Fluorescent Protein expression 24 h post-transfection. Original magnification: 100x

On the other hand, the gene delivery efficiency presented by the amphiphilic dendrimers of Joester *et al.*[34,35] greatly varied not only with the nominal charge but also with the number of C_{12} chains carried by dendrimers. In this case, the dendrimer showing the higher transfection efficiency was neither the one with less C_{12} chains nor the one with more C_{12} chains. It seems then that there is not a universal trend in transfection efficiency when hydrophobic moieties are associated to cationic polymers. Specially comparing our results with the results of Takahashi *et al.*, based on the proximity of both systems (dendron/hydrophobic tails *vs* dendrimer/hydrophobic tails), we believe that the architecture of the nucleic acid carrier plays an important role in the process. Of course that variables such as charge, size, and flexibility of the carrier must not be forgotten, as well as variables related with experimental conditions (serum presence, type of cells used, etc).

Cell viability was evaluated simultaneously to transfection experiments using the rezasurin reduction assay (Figure 8B). The less lipophilic functionalized dendrimers (La1-G5, La2-G5 and My1-G5) were as cytotoxic as G5 dendrimers whereas the more lipophilic functionalized dendrimers (My2-G5, Pa1-G5 and Pa2-G5) presented a cytotoxicity similar to SF (more toxic than G5, at least when using MSCs). Cell viability however remained above 70% in all situations and cytotoxicity results, per se, do not explain the accentuated decrease in transfection efficiency observed for the more lipophilic dendrimers. Studies on the cytotoxicity of vectors alone (not complexed with pDNA) were also performed (Figure 10). As expected, cytotoxicity was dependent on concentration but, till 20 $\mu g.ml^{-1}$ (the concentrations used in the present study were included in this range), all the systems displayed a high level of cell viability (>70%). For higher concentrations, the presence of the hydrophobic moieties had a strong effect on dendrimer cytotoxicity revealing the destabilizing nature of lipids over biological membranes[46].

Several barriers to an efficient gene delivery are described in the literature[17-21]. These include vector (or complex) cytotoxicity, complex entrance into the cell, entrapment of the complex and degradation of pDNA inside endo-lysosomes, release of pDNA from the complex, pDNA diffusion inside cytoplasm, pDNA degradation in cytoplasm, and pDNA nuclear translocation. At this point, it is clear that for the developed systems the extent of gene expression was not dependent on pDNA cellular uptake (for which the $-CH_2-$ content acted in an opposite direction) neither on cytotoxicity.

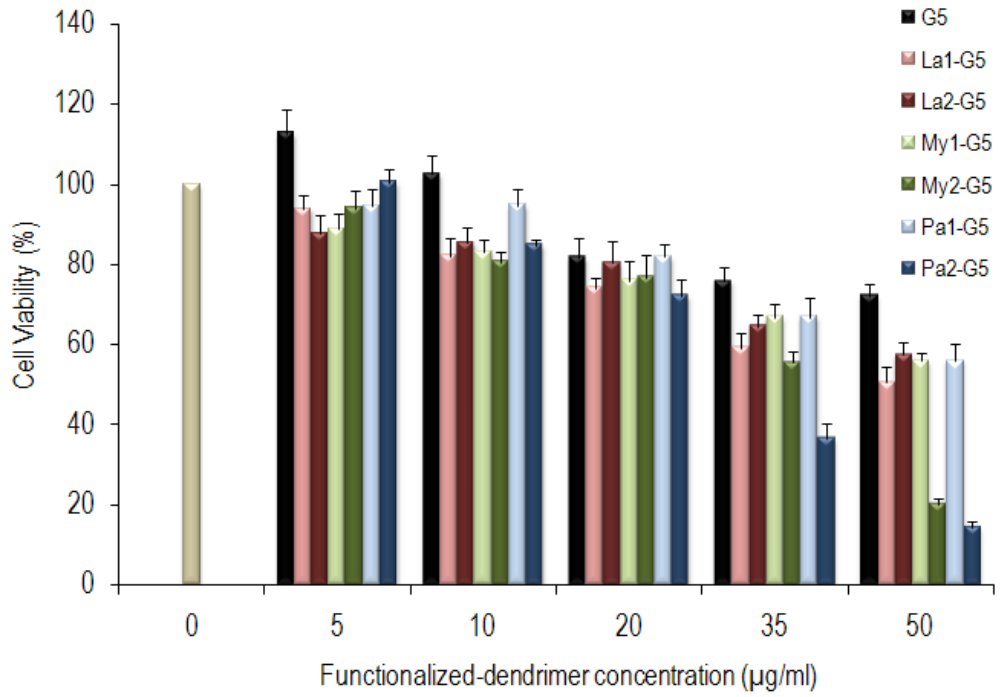

Figure 10. Cytotoxicity evaluation of the functionalized dendrimers. Each data point represents the mean ± s.e.m. of two independent measurements.

Furthermore, the studies related with the stability of complexes in the presence of serum have not shown significant differences among the vectors (all are shown to confer resistance against nucleases degradation). Also, once separated from the vector, pDNA degradation in cytoplasm should be similar in all cases. Likely, without discarding other possibilities, the higher gene expression values obtained with La1-G5 and La2-G5 vectors can be related with an easier release of pDNA from their complexes. In their study, Abbasi *et al.*[45] showed that poly(L-Lysine) polymers possessing a high level of hydrophobicity were less susceptible to dissociation. Indeed, although a fast complex dissociation in cytoplasm might facilitate pDNA degradation by nucleases, pDNA must become accessible to the transcriptional machinery in the nucleus in order to be translated in the corresponding protein. The higher gene expression values obtained with La1-G5 and La2-G5 vectors can also be due, in case the dissociation of the complexes only occur in the vicinity of the nucleus, to a higher diffusion mobility of the complexes in the cytoplasm (complexes formed between La1-G5 and La2-G5 and pDNA were shown to be smaller than complexes formed with the other vectors). Endo-lysosome disruption (by interference with the lipids in its membrane) is not expected to be facilitated for La1-G5 and La2-G5 vectors in comparison with the other systems due to their lowest -CH_2- content.

Anyway, the difficulty in nuclear translocation of pDNA is present in all situations. Fluorescence microscopy images clearly show a high accumulation of pDNA (or its complexes) at nucleus periphery after transfection. For nuclear translocation, due to the high size of the pDNA molecule, the disassembly of the nuclear envelope must occur and this will only happen during mitosis. Strategies must, then, be developed to overcome this barrier in slowly dividing cells, as is the case of MSCs. For now, based on the very good capacity of this new family of gene delivery vectors to mediate internalization of nucleic acids, we hypothesize that these systems may be explored for RNA delivery (including antisense RNA, small interfering RNA and micro RNA) since the action site of these molecules is in cytoplasm. Presently, ongoing work at our laboratory is devoted to the application of these new vectors for gene silencing. Further experiments are in progress to understand the mechanism of gene delivery using this family of vectors.

4. CONCLUSIONS

Generation 5 PAMAM dendrimer was successful functionalized at its surface with hydrophobic alkyl chains that varied in length and number. New gene delivery vectors were then synthesized which showed ability to neutralize, bind and compact pDNA, as well as to confer it protection against serum nucleases. Complexes formed between the vectors and pDNA presented sizes and ζ-potential values that, respectively, increased and decreased with the length of the hydrophobic tails. *In vitro* MSCs culture experiments revealed a remarkable capacity of these vectors for internalizing pDNA with very low levels of cytotoxicity, being this effect positively correlated with the -CH_2- content present in the hydrophobic moiety. Gene expression was also enhanced using the new vectors but, in this case, the higher efficiency was shown by the vectors containing the smallest hydrophobic chains – La1-G5 and La2-G5 functionalized dendrimers.

This new family of gene delivery vectors can have important applications in gene delivery and, hence, in tissue engineering and regeneration applications where MSCs are often used. Its capacity for internalizing nucleic acids can also be explored for RNA delivery based therapies.

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CHAPTER V.

Receptor-Mediated Gene Delivery using PAMAM Dendrimers Conjugated with Peptides Recognized by Mesenchymal Stem Cells

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ABSTRACT

Mesenchymal stem cells (MSCs) can differentiate into multiple cell types, including cells from the osteogenic, chondrogenic, myogenic and adipogenic lineages. This property makes them suitable candidates for the delivery of therapeutic genes, being necessary the development of efficient and safe vectors to attain that purpose. Moreover, specific transfection of MSCs may be required to avoid unwanted side effects in other tissues. We have synthesized and characterized a novel series of gene delivery vectors based on poly(amidoamine) (PAMAM) dendrimers functionalized with peptides displaying high affinitiy to MSCs. Novel gene delivery vectors were designed that exhibit low cytotoxicity, receptor-mediated gene delivery into MSCs and transfection efficiencies superior to those presented by native dendrimers and by Superfect® (a commercially available gene delivery system).

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1. INTRODUCTION

Bone marrow derived mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into a variety of cell types, thus being of key importance in the field of tissue regeneration and engineering[1,2]. When devising strategies for therapeutic applications, the introduction of exogenous genes in these cells is often desirable, as their expression can influence cell mitosis and differentiation. For example, MSCs isolated from a patient (autologous cells) can be propagated and transfected *in vitro* within a scaffold and using genes encoding for proteins that promote osteogenesis (such as BMP- 2). After, the construct can be implanted in a patient's skeletal defect to help bone regeneration. Viral systems, because of their sophisticated machinery, are by far the most effective nucleic acids delivery carriers but, besides being refractory to repeated infections, may raise safety problems such as acute toxicity, immunogenicity, and oncogenicity[3-5]. Non-viral systems, on the other hand, have been shown to present a limited success in primary cell transfection, as is the case of MSCs, being necessary to develop efficient and safe vectors to attain that purpose[6, 7]. Additionally, specific transfection of MSCs may be required to avoid unwanted side effects in other tissues, making the design of gene delivery vectors for cell targeting an important challenge.

Herein, we report the synthesis, characterization and evaluation of novel gene delivery vectors based on poly(amidoamine) (PAMAM) dendrimers conjugated to peptides recognized by receptors at MSCs membrane. Indeed, very recently, we have demonstrated that PAMAM dendrimers (generations 5 to 7), although showing low transfection efficiency, were able to deliver the hBMP-2 gene into MSCs, thus promoting *in vitro* osteogenesis[8]. We then hypothesized that by functionalizing dendrimers with peptides recognized by receptors at MSCs membrane, transfection efficiency could be improved and MSCs targeting could be achieved. A low affinity MSCs binding (LAB) peptide and a high affinity MSCs binding (HAB) peptide were used in the studies, based on a previously published patent[9]. With this biomimetic approach, novel gene delivery vectors were designed that exhibit low cytotoxicity, receptor-mediated gene delivery into MSCs and transfection efficiencies superior to those presented by native dendrimers and by Superfect® (SF), a commercially available gene delivery system made of partially degraded PAMAM dendrimers.

2. MATERIALS AND METHODS

2.1. Materials and reagents

Generation 5 poly(amidoamine) PAMAM dendrimers (G5) ethylenediamine-cored were obtained from Dendritech Inc. (USA). Cysteine-modified low affinity binding and high affinity binding peptides (LAB- and HAB-peptides) were synthesised with a purity of 80-90% and characterized by HPLC and MS at the University of Virginia Biomolecular Research Facility (Charlottesville, Virginia, USA). Plasmid DNA (pDNA) encoding enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) with a cytomegalovirus promoter (CMV) was generously provided by Prof. Tatiana Segura (Dep. of Chemical and Biomolecular Engineering, UCLA, USA). The plasmid was purified from E.coli cultured overnight using the GenElute™ HP Endotoxin-Free Plasmid Megaprep Kit and stored in ultrapure water at -20ºC. All other reagents used, if not specified, were obtained from Sigma-Aldrich Co. and used without further purification. Cell culture dishes were from Nunc.

2.2. Experimental determination of primary amine group content of dendrimers

The primary amine group content of G5 PAMAM dendrimers was determined by spectrophotometry after reaction of the free amine groups with TNBS as described in the literature[10], and using glycine as standard. A total of 115 amine groups was attributed to each dendrimer, this value being in agreement with the Mass Spectrometry data furnished by Dendritech Inc.. Based on this result, the TNBS method was further used to calculate the concentration of dendrimers (also of functionalized dendrimers) when needed. In both cases the standard solutions and the sample solutions were serially diluted in 0.1 M sodium tetraborate to a final volume of 1 ml. To each standard and sample 25 µl of TNBS (0.03 M) diluted in water was added. After 15 min at RT, absorption was measured at 420 nm in a GBC-Cintra 40, UV-Visible spectrophotometer.

2.3. Synthesis and characterization of peptide-functionalized G5 PAMAM dendrimers

In a first step, G5 PAMAM dendrimers (Figure 1, compound 1, C1) were substituted with various amounts of the bifunctional 3-(2-pyridyldithio)propionic acid N-hydroxysuccinimide ester (SPDP), as described earlier by Szoka *et al*.[11]. G5 PAMAM dendrimers (0.50 μmol) in 2 ml buffer (0.25 M NaCl, 0.1 M phosphate, pH 8) were mixed with SPDP (1-4 μmol) dissolved in 250 μl ethanol. The reaction was carried out at RT and under a nitrogen atmosphere. Several compounds were obtained (compounds 2, C2) by varying the molar ratio dendrimer/pyridyldithiol (PDP) (1/2, 1/4 and 1/8). The mixtures were allowed to react for 3 h, at RT, under nitrogen and with continuous stirring. Afterwards, low molecular weight products were removed by gel permeation chromatography on a PD-10 column (GE Healthcare) equilibrated in 0.15 M NaCl, 0.1 M phosphate buffer, pH 7.4. The fractions containing dendrimers linked to PDP were pooled, concentrated, snap frozen in liquid nitrogen and stored at -80 ºC. The content in C2 was estimated by TNBS assay as described previously. The degree of modification with the SPDP linker was evaluated spectrophotometrically at 343 nm by the release of pyridine-2-thion (compound 4, C4) after reduction of an aliquot with excess dithiothreitol (DTT, 0.1 M). As calibration standards, known concentrations of SPDP solutions were treated with 0.1 M DTT under the same conditions, and the changes in absorbance were used as a measure of pyridine-2-thion (C4) in solution. In a second step, cysteine-modified LAB and HAB peptides were dissolved in acetic acid (10% v/v) and mixed, under nitrogen, with C2 diluted in 500 mM phosphate buffer, pH 8. An excess of peptide (1.8- to 2.2-fold) to PDP present in C2 was used to increase reaction yields. After 3 h at RT, the amount of released pyridine-2-thion (C4) was measured at 343 nm to determine the extent of reaction. Peptide-functionalized dendrimers (C5) were purified by gel filtration using PD-10 columns equilibrated in 0.5 M NaCl, 20 mM sodium acetate, pH 5. Conjugates were applied to the column and the void fractions containing the conjugates were dialyzed against phosphate buffered saline (PBS) solution, pH 7.2, using dialysis tubes with a molecular weight cut-off of 10 kDa (Spectrum Laboratories) for 3 days. After dialysis, conjugates were sterile filtered and aliquots were snap frozen in liquid nitrogen and stored at -80ºC. Conjugates were characterized by ^{1}H NMR using D_2O as solvent in a Bruker 400 MHz Avance II^{+} NMR spectrometer.

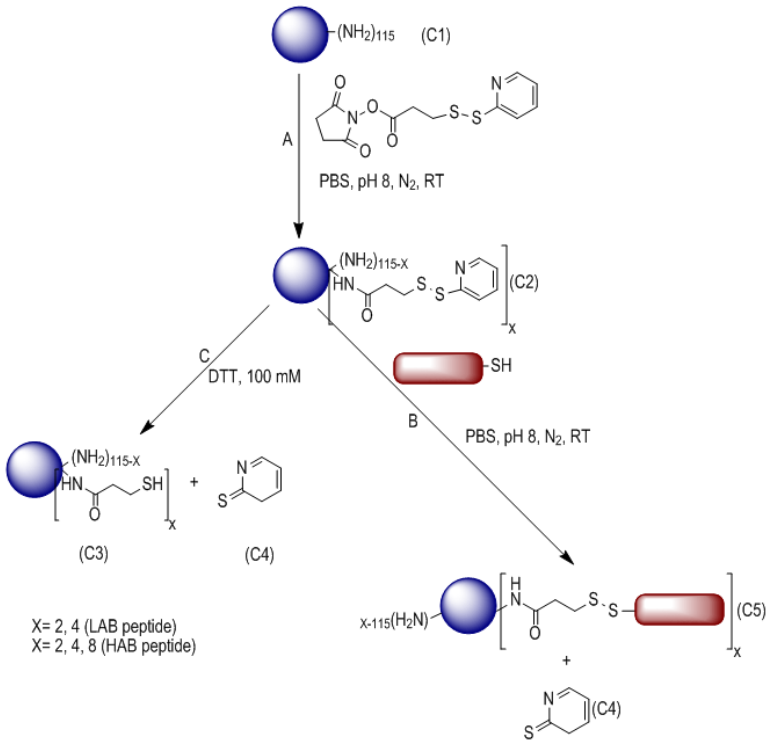

Figure 1. The two-step reaction for the synthesis of peptide-functionalized G5 PAMAM dendrimers (pathway A+B), and for indirect estimation of the number of peptide units by spectrophotometry (pathway A+C). Note: The amine content of G5 PAMAM dendrimers (115 amines/dendrimer) was previously determined experimentally

2.4. Polyplex preparation

Peptide-functionalized dendrimers/pDNA complexes were prepared at several N:P ratios by mixing equal volumes of the conjugate solution and the pDNA solution. Conjugate and pDNA concentrations varied according to the experiments. Hepes-buffered Glucose (HBG, Hepes 10 mM, Glucose 5% w/w, pH = 7.1) was always used to prepare these solutions unless otherwise stated. Polyplex solutions were vortexed gently and allowed to incubate for 20 min at room temperature prior to experiments. The generation 5 of PAMAM dendrimers was always used as a control. Superfect (Qiagen, Germany) was also used as a reference in gene delivery experiments. Superfect polyplexes were prepared according to the manufacturer's instructions for transfection of primary cells.

2.5. Agarose gel electrophoresis retardation assay

Gel electrophoresis in agarose gels was carried out at 75 V. Agarose gel (0.7 % w/v) containing ethidium bromide (0.05 μl.ml^{-1}) was prepared in Tris-acetate-EDTA buffer. Polyplex solutions were prepared at different N:P ratios, as described above, using 1 μg of pDNA diluted in 50 μl of buffer and 2.5 μl of Blue Loading Buffer (Fermentas, Germany) were added before the samples were subjected to gel electrophoresis. Functionalized dendrimers-pDNA interaction is shown by a lack of migration of the pDNA in the electrophoretic field.

2.6. Pico green intercalation assay

200 μl of polyplex solutions at different N:P ratios were prepared as described above using 0.1 μg of pDNA and an adequate amount of peptide-functionalized dendrimer, both diluted in 100 μl of Hepes-buffered saline (HBS, 10 mM HEPES, 150 mM NaCl, pH 7.0). Then, 200 μl of PicoGreen (PG, Molecular Probes) reagent, diluted in Tris-EDTA buffer (TE, 10 mM Tris, 1 mM EDTA, pH 7.5), was added and mixtures were further incubated for 5 min. Three independent experiments were performed. PG fluorescence (λ_{ex} = 485 nm, λ_{em} = 535 nm) was measured using a microplate reader (model Victor^3 1420, PerkinElmer). The % relative fluorescence (%F) was determined using the following equation:

$$\%F = 100 \times \frac{F_{sample} - F_{blank}}{F_{DNAonly} - F_{blank}}$$

2.7. Dynamic light scattering and zeta potential measurements

The size of the polyplexes was measured at 633 nm on a dynamic light scattering instrument (Zetasizer Nano ZS, Malvern Instruments). Solutions (100 μl) of polyplexes were prepared at a N:P ratio of 5, as described above, using 5 μg of pDNA diluted in 50 μl HGB. The solutions were then diluted by adding 700 μl of HGB. Particle sizes were determined for these initial solutions at room temperature with a detection angle of 173°. Zeta potential measurements

were performed using the same instrument with a detection angle of 17°. Zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions. The data presented are means of three independent sample measurements.

2.8. Isolation and culture of rat bone marrow-derived mesenchymal stem cells

Rat bone marrow-derived MSCs were isolated from long bones of 8-week-old male Wistar rats. Following euthanasia by pentobarbital 20% (v/v), femora were aseptically excised, cleaned of soft tissue, and washed in PBS. The metaphyseal ends were cut off and the marrow was flushed out from the midshaft with 5 ml of α-Minimum Essential Medium (α-MEM) using a 23-Gauge needle and syringe. The cells were centrifuged (600 g, 5 min), suspended in fresh medium containing 10% heat-inactivated foetal bovine serum (FBS, Gibco), 50 $\mu g.ml^{-1}$ ascorbic acid, 100 $U.ml^{-1}$ penicillin and 100 $\mu g.ml^{-1}$ of streptomycin - the basic medium - and seeded in 75 cm^2 flasks. After removal of non-adherent cells and medium exchange at day 3, cells were harvested at day 7 by trypsinization, and used in subsequent experiments.

2.9. Cellular uptake studies by fluorescence-activated cell sorting (FACS)

Cells were seeded in 12-well plates at a density of 1.25×10^4 $cell.cm^{-2}$ and incubated in basic medium at 37ºC, 5% CO_2, for 24 h, to yield a cell confluency of around 60-70%. Prior to polyplex formation, pDNA was labelled with PicoGreen dye (Molecular Probes) according to the manufacturer's directions. Polyplex solutions (100 µl) were prepared at a N:P ratio of 5 as previously described. Cells in 0.5 ml of basic medium were then transfected using 100 µl of the polyplex solution (2 $\mu g.cm^{-2}$ of pDNA was used). The cells were incubated with each solution for 2 h and then rinsed twice with PBS. The extracellular fluorescence associated with cell surface-bound nanoparticles was quenched with 0.4% (w/v) TB for 5 min. The cells were trypsinized, pelleted, and resuspended in 400 µl of PBS containing 2% FBS for FACS analysis (Cytomics FC500, Beckman Coulter). Twenty thousand events were collected in triplicate for each sample. Gating and analysis was performed using CXP software analysis program, using PG-labelled naked pDNA transfected cells as the primary negative control. The positive fluorescence level

was established by visual inspection of the histogram of the negative control, such that less than 1 % of positive cells appeared in the positive region. Results are expressed as the percentage of fluorescent cells and the fluorescence intensity per cell (the mean value). Three independent experiments were performed.

2.10. Intracellular trafficking of pDNA

Fluorescence microscopy was used to study the intracellular trafficking of pDNA. Prior to polyplex formation, pDNA was labelled with RITC by a slight modification of a reported method[12] Briefly, 0.1 mg of pDNA diluted in 297 μl of sodium carbonate-buffered solution (0.1 M, pH 9.0) was mixed with 3 μl of RITC solution (100 mM solution prepared in dimethyl sulfoxide) at room temperature, for 3 h. The RITC-labelled pDNA was separated from residual RITC by gel filtration using a PD-10 column (GE Healthcare), followed by ethanol precipitation to obtain the RITC-labelled pDNA. Twenty-four hours prior to transfection, cells were seeded at a density of 1.25×10^4 cell.cm^{-2} in 24-well plates containing collagen-treated cover slips. Before contact with polyplexes, medium was exchanged for fresh basic medium. Transfection was carried out with complexes prepared at an N:P ratio of 5 using 1 μg.cm^{-2} RITC-labeled pDNA. The distribution of pDNA inside cells was analyzed 2 and 4 h after transfection. Thirty minutes prior, the acidic late endosome and lysosome compartments were stained with the addition of LysoSensor DND-189 dye (Molecular Probes) to the medium at a final concentration of 1 μM. After quenching with 0.4% (w/v) TB, cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde prepared in PBS at RT for 10 min. Cell nuclei were then stained using a 300 nM 4',6-diamidino-2-phenylindole (DAPI) solution for another 10 min. Cells were washed several times with PBS and stored at 4ºC (protected from light). Fluorescence images were acquired using a Nikon Eclipse TE 2000E inverted microscope equipped with a 100x NA 0.5-1.3 Plan Fluor objective.

2.11. Gene delivery studies

Gene delivery was studied based on reporter gene expression (Luciferase, Luc, and Enhanced Green Fluorescent protein, EGFP). *Expression of the Luc gene:* Two independent

experiments were performed and all samples were performed in triplicate. Cells were seeded at 1.25×10^4 cell.cm^{-2}, in 24-well plates, 24 h prior to transfection. At the time of transfection, cells reached 60-70% confluency. Before contact with polyplexes, medium was exchanged for 0.5 ml of fresh basic medium. Polyplex solutions (100 μl) were then added to the cells and, after 4 h, the culture medium was again replaced with fresh medium. Transfection was carried out at a N:P ratio of 5 and using 1 and 2 μg.cm^{-2} pDNA. Non-transfected cells and cells transfected with naked pDNA were used as negative controls. At different time points (48, 72 and 96 h) after transfection, the media was removed and the cells were washed with PBS solution and treated with 100 μl reporter lysis buffer (Promega). Cell lysates were analysed for luciferase activity with Promega's luciferase assay reagent in triplicate (following the supplier's instructions). For each sample, the microplate reader (model Victor^3 1420, PerkinElmer) was set for 3 s delay with signal integration for 10 s. The amount of protein in cell lysates was determined using the bicinchoninic acid assay (BCA assay) with bovine serum albumin as a standard. The gene delivery efficiency of each sample was characterized by firefly luciferase expression and denoted as relative light units (RLU). Specific receptor-mediated gene delivery was confirmed by saturating cell receptors with the HAB peptide prior to transfection. Cells were incubated with a 100 μM peptide solution for 1 h. Subsequently, cells were washed twice with PBS and fresh basic medium was added. Polyplexes based on HAB peptide functionalized dendrimers were then added (a pDNA concentration of 1 μg.cm^{-2} was used in these assays) and allowed to incubate for 4 h. Luciferase activity was assessed 48 h post-transfection.

Expression of the EGFP gene: Enhanced Green Fluorescent protein expression studies were carried out as mentioned above for the Luc gene expression. Twenty-four hours after transfection, cells were observed with an inverted fluorescence microscope (Nikon Eclipse TE 2000E) equipped with a cold Nikon camera. Digital image recording and image analysis were performed with the NIS Elements Advanced Research (version 2.31) software.

2.12. Cytotoxicity studies

The cytotoxicity of the gene delivery vectors (alone) and of the polyplexes they form with pDNA was studied. Cytotoxicity was evaluated by determining the percentage of cell viability (in respect to unexposed cells) using the rezasurin reduction assay that establishes a correlation

between the cellular metabolic activity and the number of viable cells in culture[13]. *Gene delivery vector cytotoxicity:* Cell viability was studied as a function of the gene delivery vector type and concentration. Cells were seeded in 96 well-FluoroNunc plates at a density of $3x10^4$ cell.cm^{-2}. After 24 h, medium was replaced with fresh basic medium and 10 µl of each polymer (diluted in HBG) was added to achieve the final desired concentration. After 4 h, the medium was exchanged for fresh medium containing 0.1 mg.ml^{-1} rezasurin and incubated for another 4 h. Resorufin fluorescence (λ_{ex} = 530 nm, λ_{em} = 590 nm) was measured in a microplate reader (model Victor^3 1420, PerkinElmer).

Polyplex cytotoxicity: The information about polyplex cytotoxicity was obtained during the experiments performed to study the expression of the Luc gene (described above), being cell viability evaluated 24 h post-transfection

2.13. Statistics

Statistical analyses were performed using GraphPad Prism 5.0 for Windows. Results are reported as mean± standard error of mean (s.e.m). Unpaired, Student's t-test and 2-way ANOVA with Bonferroni Post Hoc test were used to assess the statistical differences between the group means.

3. RESULTS AND DISCUSSION

Generation 5 PAMAM dendrimers (G5) with amine termini (C1) were conjugated to LAB and HAB peptides via a two-step method using the 3-(2-pyridyldithio) propionic acid N-hydroxy-succinimide ester (SPDP), a heterobifunctional crosslinker which contains both amine- and sulfhydryl-reactive groups (Figure 1). For linkage, a cysteine residue was present at the end of both amino-acid sequences - NSMIAHNKTRMHGGGSC (LAB peptide) and SGHQLLLNKMPNGGGSC (HAB peptide). During the synthesis, the concentration of dendrimers (also of functionalized dendrimers) was determined by spectrophotometry, based on primary amine content. The degree of functionalization was under stoichiometric control and was indirectly estimated by spectrophotometric quantification of pyridine-2-thion (C4) that is released after reduction of the

dendrimer-PDP intermediate (C2) with excess of dithiothreitol (DTT) and after reaction of C2 with the peptides (Table 1).

Table 1. Average number of PDP and of peptides per dendrimer, respectively, after reactions A and B. *Theoretical value based on the molecular weight of dendrimers provided by the supplier.

Peptide	Average number of PDP per dendrimer (after reaction A)	Average number of peptides per dendrimer (after reaction B)	Mol. weight* (u.m.a.)
NSMIAHNKTRMHGGGSC LAB peptide	2.1	2.1	31008
	4.0	3.9	34866
SGHQLLLNKMPNGGGSC HAB peptide	2.2	2.1	30830
	4.1	4.1	34510
	8.2	8.1	41870

The conjugates were further characterized by ^{1}H NMR spectroscopy (Figure 2) using D_2O as solvent in a Bruker 400 MHz Avance II+ NMR spectrometer at RT. Figure 2 exemplifies ^{1}H NMR characterization for the conjugate G5-$(HAB)_8$. As can be seen, the ^{1}H NMR showed the appearance of new proton signals such as the imidazole protons around δ = 6.85 (s, 8H) and δ = 7.6 (s, 8H) (Histidine aminoacid), methylene protons around δ = 3.9 (CH_2, s, 64H) (Glycine aminoacid), pyrrolidine protons at δ = 2 (CH_2, s, 16H) (Proline aminoacid), and methyl protons around δ = 0.7 – 0.9 (CH_3, s, 144H) and methine protons (CH, s, 18H) at δ = 1.5 (Leucine aminoacid), thus confirming that the peptides were successfully conjugated to G5 PAMAM dendrimers in a ratio of 8:1. ^{1}H NMR spectra were all in accordance with the results shown in table 1.

Conjugates with two (G5-$(HAB)_2$ and G5-$(LAB)_2$) and four (G5-$(HAB)_4$ and G5-$(LAB)_4$) peptide arms were first prepared. As previous gene delivery experiments revealed promising results with conjugates bearing the HAB peptide, a conjugate with eight HAB peptide arms (G5-$(HAB)_8$) was also synthesized to better evaluate the influence of the number of attached peptides on the behavior of conjugates as gene delivery vehicles.

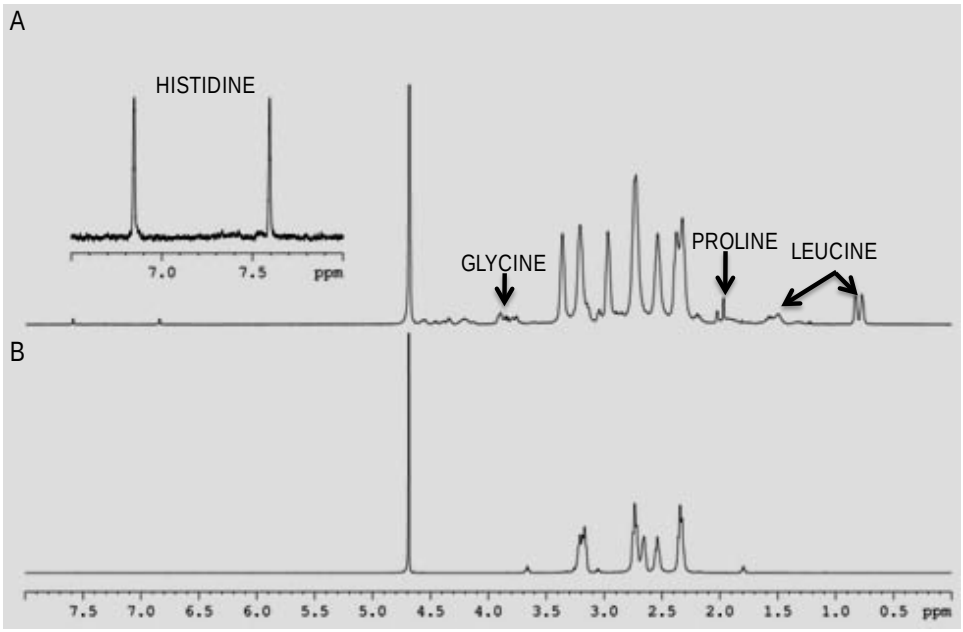

Figure 2. ^{1}H NMR of (A) G5-$(HAB)_8$ and of (B) G5 native dendrimer. The inset shows the imidazole proton peaks from the amino acid histidine.

Peptide-functionalized dendrimers were first investigated for their ability to bind, neutralize and compact plasmid DNA (pDNA). Plasmid DNA encoding for enhanced Green Fluorescent Protein and Firefly Luciferase (pEGFPLuc, 6.4 kb) was used. Prior to all studies, conjugates were mixed with pDNA at several N:P ratios in HBG for polyplex formation. Agarose gel retardation assays (Figure 3A) revealed that binding to and charge neutralization of pDNA occurred at N:P ratios of 1 and higher when native dendrimers were used, whereas for conjugated dendrimers the hampering of pDNA migration occurred at N:P ratios of 2 and higher. By using the PicoGreen (PG) dye (Figure 3B), which, when not bound to DNA has virtually no fluorescence, we concluded that complete pDNA packaging was achieved at a N:P ratio of 2 for the native dendrimer and at a N:P ratio of at least 4 for the conjugated dendrimers. Based on these results, a N:P ratio of 5 was selected for further experiments. At this ratio, dynamic light scattering (DLS) and ζ-potential measurements were performed to examine the size and colloidal stability of the formed polyplexes (Figures 4A and 4B).

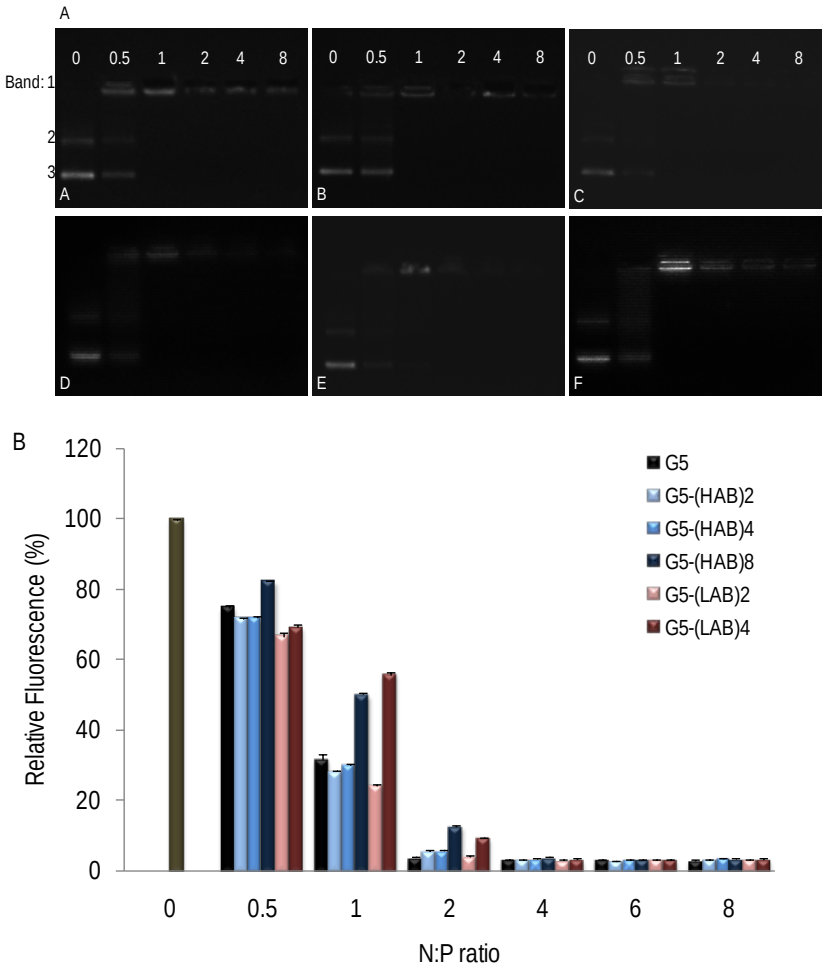

Figure 3. (A) Agarose gel retardation assay results for N:P ratios ranging from 0 (pDNA only) to 8: (a) G5; (b) G5-$(HAB)_2$; (c) G5-$(HAB)_4$; (d) G5-$(HAB)_8$; (e) G5-$(LAB)_2$; (f) G5-$(LAB)_4$. Binding is shown by the inhibition of pDNA electrophoretic mobility (band 1). Bands 2 and 3 show the relaxed and supercoiled forms of pDNA, respectively. (B) PicoGreen assay. The results are reported as the relative percentage of PG fluorescence, where 100% intensity was observed for a N:P of 0 (pDNA only). Results are expressed as the mean ± s.e.m obtained from three independent experiments.

Conjugation of peptides to native dendrimers caused a significant increase ($p<0.05$) in polyplex size which, nevertheless, ranged between 110 and 160 nm in diameter. The polydispersity indices (PDI) were only slightly increased by the incorporation of peptides, revealing that the level of homogeneity in the samples remained nearly constant. The incorporation of peptides also resulted in a significant decrease (about 40%, $p<0.001$) in ζ-potential values. Even if this fact can be interpreted as an increase in colloid hydrophobicity and concomitant instability that may lead to aggregate formation, it is also challenging since the positive, low-charged polyplexes obtained might establish reduced interactions with counter ions

and plasma proteins, which are known to promote blood clearance and interfere with transfection *in vivo*[14,15]. Taken together, results suggest that the peptide arms around dendrimers have a shielding effect over primary amines, reducing polyplexes ζ-potential, conducting to the need of using higher N:P ratios for full pDNA compaction and increasing polyplex size. A similar effect was reported using poly(ethylene glycol) chains linked to PAMAM dendrimers[16].

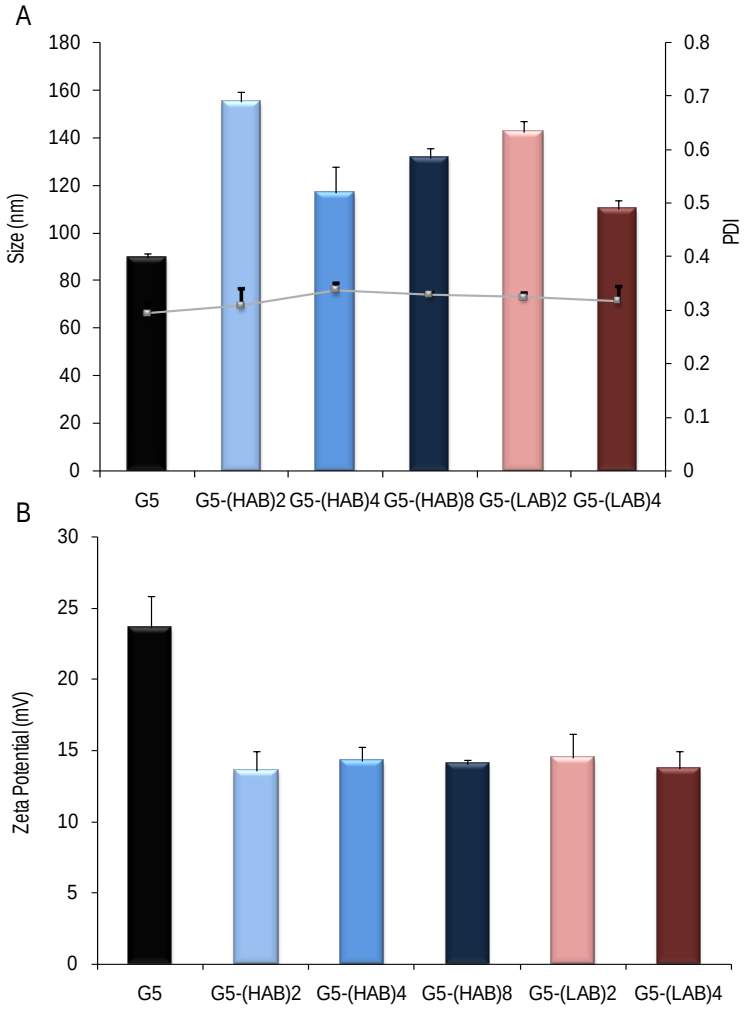

Figure 4. (A) Polyplex size (mean diameter) and polydispersity indices assessed by DLS, and (B) polyplexes ζ-potential. Results were obtained for a N:P ratio of 5. Results are expressed as the mean ± s.e.m obtained from three independent experiments.

PicoGreen-labeled pDNA was used to compare polyplex uptake by rat bone marrow derived MSCs via fluorescence-activated cell sorting (FACS) (Figure 5). The experiments were done under the same conditions used in the following transfection assays. Cellular uptake was

studied in the presence of 10% (v/v) serum, at a N:P ratio of 5 and using a seeding density of $1.25x10^4$ cell.cm^{-2} and 2 μg.cm^{-2} pDNA. After 2 h of contact between polyplexes and cells, both the number of cells positive for PG-labeled pDNA and the amount of pDNA delivered per cell achieved with HAB peptide-functionalized dendrimers (G5-$(HAB)_2$ and G5-$(HAB)_4$ were significantly higher ($p<0.05$) than those obtained with native dendrimers and similar to those reached using SF. However, pDNA internalization using G5-$(HAB)_8$ was lower possibly due to the fact that this vector interacts with a higher number of receptors at the cell membrane causing a saturation-like phenomenon and hampering the cellular uptake process[17] Cellular uptake values for LAB peptide-functionalized dendrimers were only slightly higher than those obtained with native dendrimers.

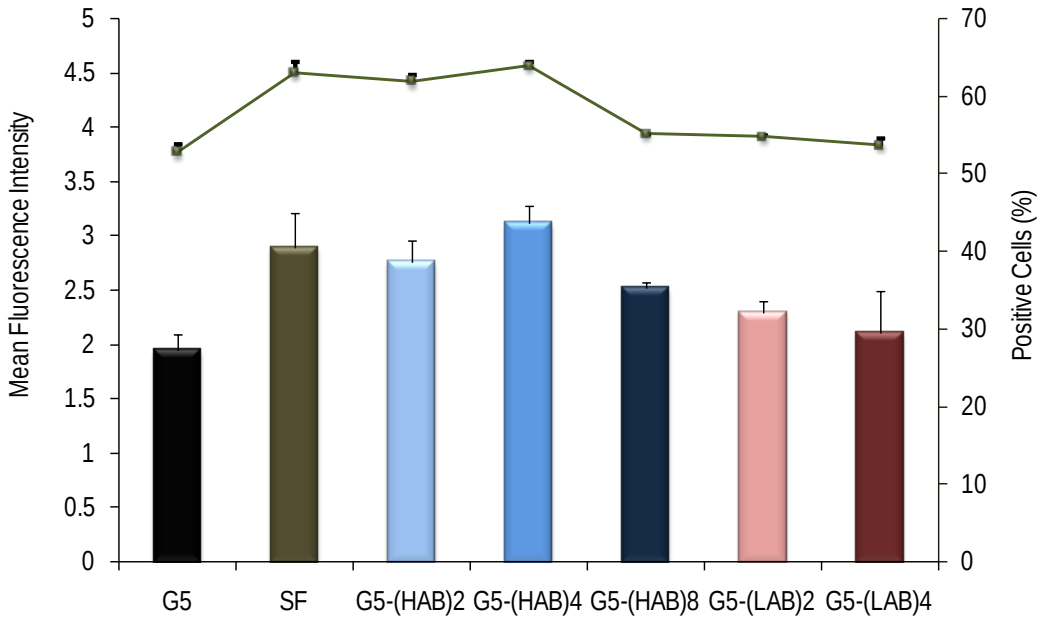

Figure 5. Polyplex uptake by MSCs after 2 h of contact. The line shows the number of cells positive for PG-labeled pDNA, whereas the bars reveal the amount of pDNA delivered per cell (displayed as the mean average fluorescence intensity). Results are expressed as the mean ± s.e.m obtained from three independent experiments.

Figure 6 shows the distribution of RITC-labeled pDNA (red) inside cells after transfection using native dendrimers and peptide-functionalized dendrimers (G5-$(HAB)_4$ and G5-$(LAB)_4$). The acidic late endosome and lysosome compartments were stained with LysoSensor Green DND-189 (green), and the nucleus with DAPI (blue). The images were obtained using a lower concentration of pDNA (1μg.cm^{-2}) to diminish blur. Two hours post-transfection, a higher accumulation of pDNA could be observed inside cells when using HAB peptide functionalized-

dendrimers. These data are in agreement with FACS results, which showed that HAB peptide-functionalized dendrimers were able to deliver a high amount of pDNA per cell.

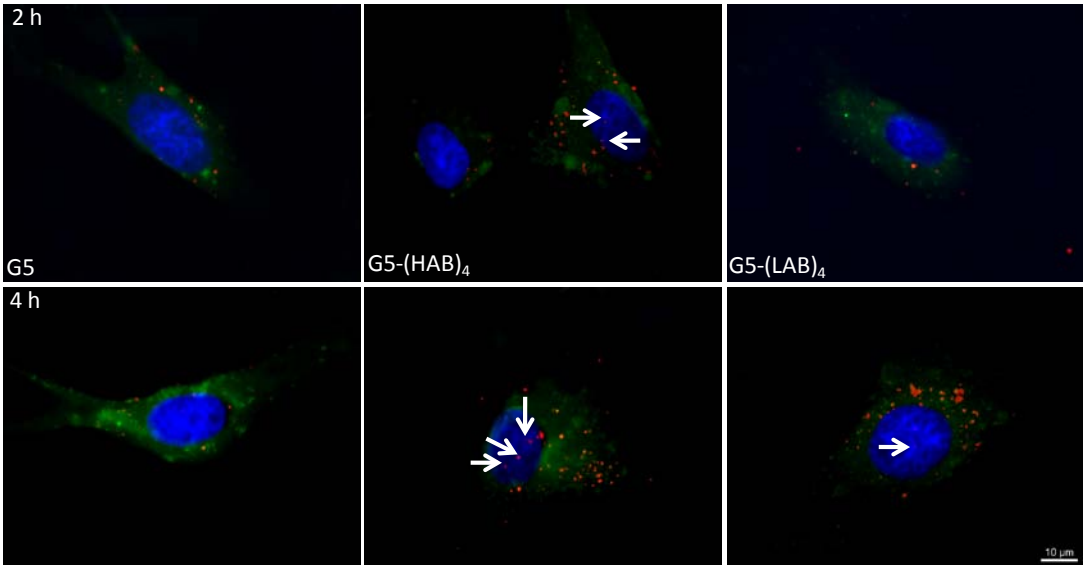

Figure 6. Cellular distribution of (RITC)-labeled pDNA (2 and 4 h post-transfection) using native dendrimers and G5-$(HAB)_4$ and G5-$(LAB)_4$ as vectors. The acidic late endosome and lysosome compartments were stained with LysoSensor Green DND-189 (green), and the nucleus with DAPI (blue). Original magnification: 1000x.

As time progressed, it was possible to observe the appearance of orange spots representing the co-localization of pDNA and endosome/lysosome compartments. pDNA co-localization with the nucleus (pink dots, white arrows) could already be observed after 2 h when HAB peptide-functionalized dendrimers were used.

To quantitatively investigate if the conjugation of peptide to dendrimers was beneficial in terms of gene delivery, rat bone marrow derived MSCs were assayed for expression of the Luc reporter gene after 48, 72 and 96 h post-transfection (Figure 7A and 7B). Results were normalized to protein content and are shown as relative light units (RLU). A transient expression of the Luc gene was obtained in all cases with HAB peptide-functionalized dendrimers containing 4 and 8 peptides per dendrimer leading to an earlier gene expression possibly related with a faster polyplex cellular uptake and pDNA trafficking to the nucleus. After 48 h, HAB peptide-functionalized dendrimers containing four and eight peptides per dendrimer presented transfection levels that were, respectively, 10 and 5.5-fold higher than those obtained with native dendrimers and 2.5 and 1.5 higher than those achieved with Superfect.

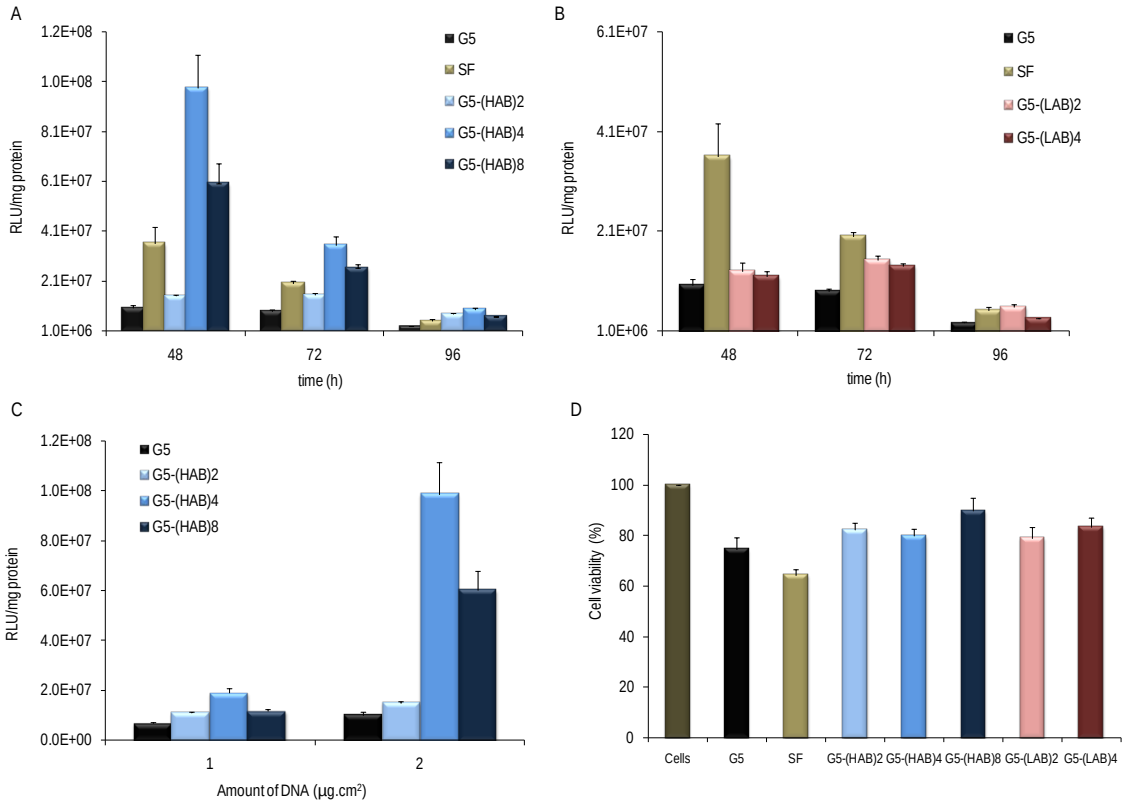

Figure 7. (A) Luc gene expression achieved with HAB peptide-functionalized dendrimers, (B) Luc gene expression achieved with LAB peptide-functionalized dendrimers, and (C) comparison of Luc gene expression 48 h post-transfection achieved with HAB peptide-functionalized dendrimers for different pDNA concentrations (1 µg.cm^{-2} and 2 µg.cm^{-2}). (D) Cytotoxicity evaluation 24 h post-transfection. Results are expressed as the mean ± s.e.m. and were obtained from two independent experiments.

Although more moderate, a positive difference could still be noticed after 72 h post-transfection between these vectors and all the others. Transfection efficiency was dependent on pDNA concentration as shown by decreasing it to 1 µg.cm^{-2} (Figure 7C). In this case, the enhancement of transfection efficiency is not so high but a positive influence of HAB peptides on gene delivery is still observed. On the other hand, the transfection efficiency observed with conjugates bearing LAB peptides was only slightly higher than that obtained with native dendrimers, reflecting the low affinity characteristics of this peptide for MSCs. Gene delivery achieved by peptide-functionalized dendrimers was also qualitatively studied, 24h post-transfection, by visualization of Enhanced Green Fluorescent Protein expression using fluorescence microscopy. Results are shown in Figure 8.

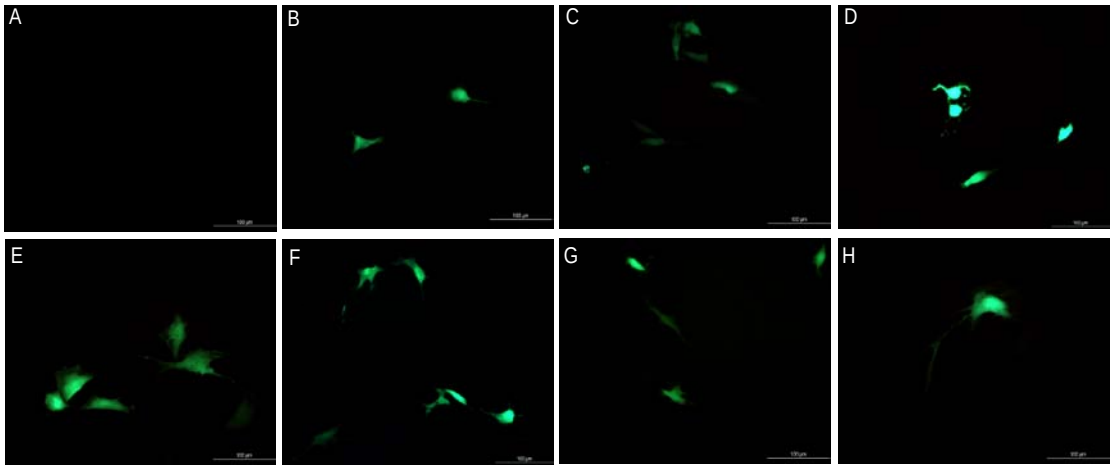

Figure 8. Fluorescence microscopy images showing Enhanced Green Fluorescent Protein expression 24 h post-transfection using: (A) naked DNA, (B) G5 PAMAM dendrimers, (C) Superfect, (D) G5-$(HAB)_2$, (E) G5-$(HAB)_4$, (F) G5-$(HAB)_8$, (G) G5-$(LAB)_2$, and (H) G5-$(LAB)_4$. Original magnification: 200 x.

Cell viability was assessed simultaneously to transfection experiments using the rezasurin reduction assay. As shown in Figure 7D, the remarkable enhancement of the Luc gene expression presented in particular by G5-$(HAB)_4$ and G5-$(HAB)_8$ vectors was accomplished with minimal cytotoxic effects to cultured cells (viability was around 80-90%). In contrast, the transfection efficiency obtained with Superfect was accompanied by a notable decrease (~40%) in cell viability. In general, polyplexes formed by native dendrimers presented a higher toxicity than those formed by the modified polymers. Studies on the cytotoxicity of vectors alone were also performed (Figure 9), revealing that functionalized dendrimers display a significantly lower toxicity than native dendrimers and SF, particularly for the highest concentrations. These facts may be explained by the partial shielding of primary amines (primarily responsible for cytotoxicity effects) achieved in the conjugated peptides.

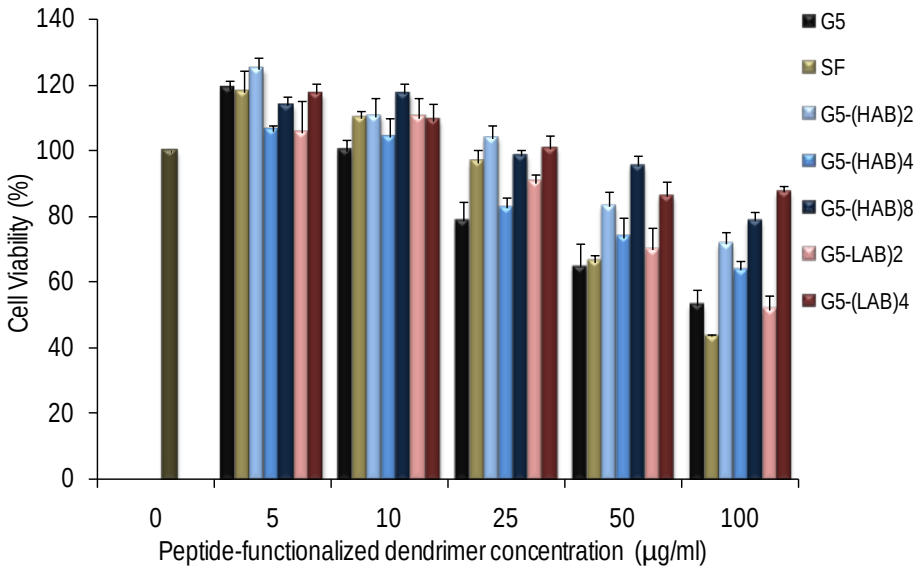

Figure 9. Cytotoxicity evaluation of peptide-functionalized dendrimers. Each data point represents the mean ± s.e.m. of two independent measurements

The receptor-mediated nature of gene delivery conducted by conjugates attached to HAB peptides was confirmed by saturating cell receptors with the peptide prior to transfection (a 0.1 mM peptide solution was incubated with cells for 1 h). In this case, the level of transfection achieved with HAB peptide-functionalized dendrimers was similar to that attained with native dendrimers (Figure 10A). No cytotoxic effect associated with the previous saturation of cell receptors with the peptide was observed (Figure 10B).

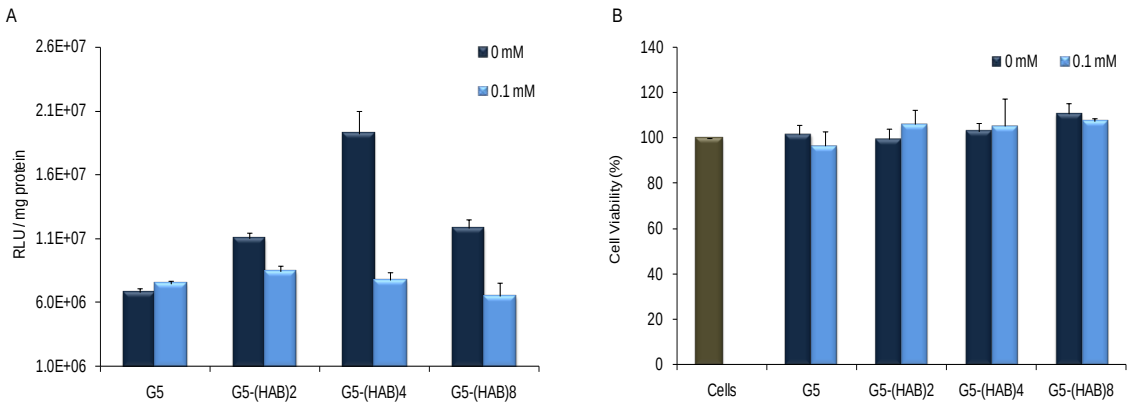

Figure 10. (A) Luc gene expression achieved with HAB peptide-functionalized dendrimers with and without saturation of cell receptors by HAB peptide prior to transfection. (B) Cytotoxicity evaluation 24 h post-transfection. Results are expressed as the mean ± s.e.m. and were obtained from two independent experiments.

In conclusion, conjugation of PAMAM dendrimers with peptides with high binding affinity for MSCs provides a mechanism for cell specific recognition, giving rise to a new family of gene delivery vectors presenting low cytotoxicity and transfection efficiencies superior to those of native dendrimers or partially degraded dendrimers. These systems represent a step forward in the gene delivery field and may have important applications in tissue engineering and regeneration

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SECTION 3.

CHAPTER VI.

CONCLUSIONS and PERSPECTIVES

CHAPTER VI.

1. CONCLUSIONS

The main aim of this thesis was to explore the *in vitro* capacity of several PAMAM dendrimer based nanomaterials to deliver genes to MSCs. From the overall work, some conclusions can be drawn/comments can be made:

(a) Throughout this dissertation, PAMAM dendrimers (with amine termini) were shown to be very interesting materials for gene delivery into MSCs. In one hand, their globular architecture and cationic nature allows them to interact with the negatively charged DNA molecules resulting in their condensation. In the other hand, their high degree of branching and multivalency characteristics allows their functionalization with biological targeting molecules.

(b) Native dendrimers are able to transfect MSCs. The generation number, the N:P ratio and the cell passage number were found to have a significant impact on transfection efficiency. By using a plasmid encoding the hBMP-2 gene, MSCs can be induced to produce BMP-2 and to differentiate towards the osteoblastic lineage. Results from chapter II clearly show that even if a low transfection level is obtained, it is sufficient for the *in vitro* osteogenic differentiation of MSCs (as shown by the analyzed osteogenic markers). Transfection in HEK 293T is much higher, confirming that the process of gene delivery is also cell type dependent.

(c) Functionalization of PAMAM dendrimers with RGD containing peptides, alkyl groups and MSCs high affinity binding peptides resulted in an improvement in the level of gene delivery in comparison with the native dendrimers and, with exception for the first case, with the commercial product SuperFect®. All these approaches aimed at establishing specific interactions with the cell membrane, either through receptors at its surface or by interfering with its constituent lipids. Vector's capacity to condense pDNA was not affected by functionalization. In general, the cytotoxicity of the functionalized dendrimers was low, being inferior to that showed by SuperFect® and having similar values to those showed by native dendrimers. Conjugation of dendrimers with RGD containing peptides (chapter III) allowed the formation of RGD nanoclusters and revealed the importance of these structures on the modulation of the gene transfer process. Alkyl chain-functionalized dendrimers (chapter IV) presented a remarkable capacity for internalizing pDNA, being this effect positively correlated with the $–CH_2–$ content present in the hydrophobic corona –

these new vectors can then be very promising for RNA delivery based therapies. Gene expression was also enhanced using the new vectors but, in this case, the higher efficiency was shown by the vectors containing the smallest hydrophobic chains. A receptor-mediated gene delivery was obtained with PAMAM dendrimers conjugated with peptides displaying high binding affinity towards MSCs (chapter V). These new multifunctional materials can be potentially used for a large variety of therapeutic applications including the delivery of drugs and genes that could help in the treatment of musculoskeletal disorders (genetic or acquired), osteoporosis and metastatic cancer.

(d) MSCs are indeed very difficult to transfect. Despite the improvements in gene delivery attained with the functionalization of dendrimers described in this thesis, the levels of gene expression are modest if we compare them with the results obtained for other types of cells (chapter II). Fluorescence microscopy images of cells after transfection with RITC-labeled pDNA always show a high accumulation of nucleic acid in cytoplasm in respect to the quantity found inside the nucleus. These results suggest that the major barriers to an efficient non-viral gene delivery is the release of pDNA from PAMAM dendrimer based vectors and/or degradation of pDNA inside cytoplasm and/or pDNA nuclear translocation.

2. PERSPECTIVES

The results presented in this thesis raise new queries and demand supplementary studies in order to extend our knowledge about gene therapy strategies making use of mesenchymal stem cells as cellular vehicles and PAMAM dendrimer based gene carriers.

From our results, as previous mentioned, it seems that the main barrier to an efficient non-viral gene delivery is the release of pDNA from PAMAM dendrimer based vectors and/or degradation of pDNA inside cytoplasm and/or pDNA nuclear translocation. More studies are then needed to address this issue, such as a detailed investigation of the trafficking of pDNA inside cells. For this, cellular localization of pDNA alone and of vector/pDNA complexes (co-localization studies of vectors and pDNA) using cell compartment biomarkers could give valuable information. Further experiments aimed at the evaluation of pDNA stability during its trafficking to cell nucleus should also be important (resistance to changes in pH, resistance to nucleases, etc.). Finally, the

physicochemical characterization of vector/pDNA complexes also deserves a profound investigation. Transmission electron microscopy and atomic force microscopy, for example, can provide information concerning the interaction between pDNA and gene delivery vectors that will help on the design of more efficient gene carriers. Concerning the low release of pDNA from dendrimers, the use of dendrimers with more flexible cores and biodegradable components might be recommendable. In order to improve the nuclear translocation of pDNA, a peptide sequence presenting nuclear translocation properties can also be fitted to dendrimers or even to the backbone of pDNA molecules.

So far the biological performance of the PAMAM dendrimer based vectors developed in this thesis was only studied using *in vitro* cell culture assays. As a next step, the potential of these vectors for gene delivery needs an *in vivo* evaluation. For an *in vivo* application several animal models and strategies can be adopted. By analyzing the literature (chapter I), a cell-mediated gene therapy approach should be the best option. Here, cells are harvested from the adult animal, genetically engineered *in vitro* to express the therapeutic gene, selected for enrichment in the cells expressing the gene of interest, seeded in a scaffold and, then, implanted at the wound site. Using this methodology, the implanted genetically engineered MSCs may enhance tissue regeneration through two mechanisms: by directly participating in tissue regeneration and by producing growth factors that will enhance the function of native cells in or around the tissue defect through paracrine signaling. Other strategies can be followed considering the advantages and drawbacks of each methodology and depending on the work's ultimate goal.

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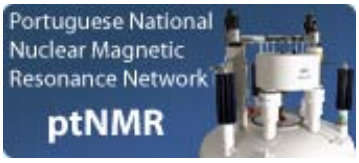

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