Tissue Engineering Scaffold Material with Enhanced Cell Adhesion and Angiogenesis from Soy Protein Isolate Loaded with Bio Modulated Micro-TiO$_2$ Prepared via Prolonged Sonication for Wound Healing Applications

Rekha Rose Koshy, Siji K. Mary, Arunima Reghunadhan, Yogesh Bharat Dalvi, Lekshmi Kailas, Nereida Cordeiro, Sabu Thomas, and Laly A. Pothen

Cite This: ACS Biomater. Sci. Eng. 2022, 8, 4896−4908

ABSTRACT: Tissue engineering is a technique that promotes healing by creating an ideal environment for endogenous cells to migrate and grow into the site of injury via a scaffold, improving regeneration and reducing the time required for in vitro cell culture. In this work, the effect of the addition of sonicated TiO$_2$ in the soy protein isolate (SPI) matrix for tissue engineering applications was studied. In comparison to adding expensive nano TiO$_2$, this method of incorporating sonicated TiO$_2$ into the SPI matrix will aid in achieving improved properties at a lower cost. The effect of the addition of sonicated TiO$_2$ on the morphological, UV transmittance, mechanical, thermal, surface energy, and hydrophilicity of SPI films was investigated. The result shows that the uniformly distributed TiO$_2$ particles successfully blocked 95% of UV light. Scanning electron microscopy revealed a significant reduction in the TiO$_2$ agglomerate size and homogeneous distribution of the same when sonication was applied instead of mechanical dispersion. A simultaneous increase of tensile strength (from 3.16 to 4.58 MPa) and elongation at break values (from 24.25% to 95.31%) with 0.5% TiO$_2$ was observed. The addition of 0.25% TiO$_2$ was found to significantly enhance the elongation at break value to 120.83%. Incorporation of micro-TiO$_2$ particles could improve the surface roughness, surface energy, and wettability of SPI films. In vitro cell adhesion studies and in vivo subcutaneous implantation studies were performed to assess the cell growth and angiogenesis of the developed film membranes. An MTT assay showed that SPI-1%TiO$_2$ film favored cell viability up to 118%, and in vivo subcutaneous implantation studies showed enhanced cell growth and angiogenesis for SPI-1% TiO$_2$ films. This SPI-TiO$_2$ film with enhanced surface properties can be used as an ideal candidate for tissue engineering applications.

KEYWORDS: SPI, TiO$_2$, cell adhesion, sonication, tissue engineering

1. INTRODUCTION

The technique, tissue engineering, is a combination of regeneration and repair of cells and tissues by incorporating various materials that are compatible with the body. A considerable amount of progress has been made in this field, both in the development of new tools and in converting
Various biomaterials are compared to functional tissue engineered products. However, conventional tissue engineering techniques, which use prefabricated scaffolds for implantation at the injury site, require a lot of time and labor. In this context, in situ tissue engineering was proposed to overcome the limitations of cell-based tissue engineering by utilizing the body’s own regenerative capabilities. A good target-specific scaffold should interact with the living cells, resulting in long-lasting survival and promoting vascularization.

The use of bioactive materials in scaffolds is found to favorably increase vascularization without the need to add an external growth factor. Titanium dioxide (TiO$_2$) is one such bioactive material that is reported to have the potential to induce angiogenesis. The ability of TiO$_2$ to promote angiogenesis is attributed to the development of reactive oxygen species radicals on exposure to light. TiO$_2$ is commonly used as a photocatalyst owing to its strong oxidizing abilities, chemical stability, super hydrophilicity, nontoxicity, sturdiness, low cost, and transparency under visible light. The majority of the reported work used nanoscale titania as a filler and is very expensive. In comparison with TiO$_2$ nano powder, TiO$_2$ microscale structures have higher surface areas, more tunable opening(pore) structures, and better photocatalytic activity. The microscale titania can be produced by the simple ultrasonication process in a probe sonicator from its macro precursor. In this sonication process, the filler is subjected to a high shear process using ultrasonic energy. Passing ultrasonic waves through the filler solution results in the formation of high pressure, which breaks the filler into a small size. Dispersion using ultrasonic waves is also being used to break the binding energy of particles without the need for any external chemical agents.

Several studies have been published on the use of TiO$_2$ nanoparticle reinforced biopolymeric matrices as biomaterials. There have been few or no reports on the use of micro-TiO$_2$ particles as frameworks for tissue engineering applications. As a result, in this study, we reinforced a protein-based polypeptide matrix with TiO$_2$ and assessed its potential for use as a biomaterial. Protein-based biopolymeric matrices have been reported to exhibit superior biological behavior when compared to polysaccharides due to the presence of polypeptide chains containing reactive amino acids. Soy protein isolate (SPI) is one such plant-based protein, which when reinforced with suitable bioactive fillers, finds excellent applicability in the biomedical field. SPI, being a naturally occurring protein can recapitulate the physiochemical and biological features of the native extracellular matrix (ECM), thereby achieving the highest level of biomimicry. Because of their inherent functional groups and nutrient ingredients, SPI-based biomaterials have been shown in studies to stimulate cell growth without the need for additional growth factors. Furthermore, SPI-based materials can promote collagen formation by fibroblasts and thus have significant wound healing potential. As a result, the effect of sonicated TiO$_2$ on the potential of SPI as a biomaterial is being investigated.

Before entering into in vivo applications with SPI-based composites, the surface biological characteristics must be thoroughly investigated. SPI-like biomaterials should exhibit not only biocompatibility and biostability but also specific surface biological characteristics that can induce positive cell responses. Surface roughness and morphology have a significant impact on the biomaterial’s surface energy, which improves tissue growth and cell responses. Surface roughness is well-thought-out to influence the reactions of adherent cells, especially in the case of body implants, and an efficient design is a rough topographic surface. Previous studies have shown that TiO$_2$ composites with high surface roughness can promote epithelial growth and attachment of fibroblasts.

This study examined the impact of sonicated TiO$_2$ on the optical, mechanical, and thermal properties of SPI films. The ability of the produced SPI-TiO$_2$ film for tissue engineering applications was investigated in particular. The effect of the addition of sonicated TiO$_2$ on the surface properties like roughness, surface energy, hydrophilicity, adsorption, and diffusion coefficient of the SPI film was studied. Also, distinct from TiO$_2$ nanopowder, TiO$_2$ microscale structures have a higher surface area, more tunable pore structures, and improved photocatalytic activity. The ability of the scaffolds to enhance cell adhesion, migration, and proliferation of endogenous cells that facilitate repair was monitored. The ability of the scaffolds to stimulate the production of cell proliferative and angiogenic factors that can enhance cell growth and vascularization was assessed using in vivo and in vitro models.
2. MATERIALS AND METHODS

2.1. Materials. TiO₂ macroparticles (rutile) were purchased from Merck. The protein isolate (SPI, 98 wt % protein) was obtained from Adeesh Agrofoods (Haryana, India). All reagents used were of analytical rating. Deionized water was used for all sample preparations.

2.2. Preparation of SPI/TiO₂ Films. The isolate powder was liquefied using 200 mL of distilled water that contained 3.6 mL of glycerol as the plasticizing agent to create the SPI solution. TiO₂ dispersions in the desired quantity were combined with the SPI solution and agitated for 10 min. 0.25%, 0.5%, 0.75%, and 1% of TiO₂ solutions previously sonicated for 1 h were added in varying amounts. TiO₂ was added very gradually while being vigorously stirred into the protein solution in order to achieve a proper dispersion.

The pH of the resulting solutions was attained to 10 ± 0.1 with 1 N sodium hydroxide. The solutions were then kept at 85 °C for 20 min, emptied onto a Teflon pan, and dehydrated at 55 °C for 12 h in a vacuum oven (Scheme 1). After drying, the films were peeled off and stored at 43 ± 2% RH and 25 ± 2 °C for 24 h prior to testing.

2.3. Appearance and Color Parameters of SPI-TiO₂ Films. The color parameters of the film sample were measured with an SC-80C colorimeter, and its L (lightness), a (redness to greenness), and b (yellowness to blueness) values were determined. L can have values from 0 (black) to 100 (pure white). a can have a value from negative (green) to positive (red), and b can assume value from negative (blue) to positive (yellow); Luchese et al.

2.4. Scanning Electron Microscope (SEM). The surface morphology of sonicated and nonsonicated TiO₂ solutions and SPI-TiO₂ films was done using a TESCAN Vega-3 scanning electron microscope (5.0 kV accelerating voltage).

2.5. Thickness Measurements. For the thickness measurement of the specimens, a screw gauge with a correctness of 0.01 mm was used. The measurement was carried out at five randomly selected points of the sample film and was measured for each specimen. The result were plotted by considering the mean values.

2.6. Intrinsic Fluorescence Spectroscopy. The fluorescence of SPI-TiO₂ films was examined at room temperature (25 ± 1 °C) using a Carry 50 Fluoro spectrophotometer (Varian, USA). Excitation and emission slits used for the experiments were 10.0 and 20.0 nm, respectively, with an excitation wavelength of 290 nm and emission spectra from 305 to 450 nm. Each sample had three scans.

2.7. Ultra Violet Spectroscopy (UV). The absorbance spectrum (200–700 nm) of the films was documented using a UV mini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). The sample was cut into a rectangular-shaped piece and placed in the spectrophotometer test cell, and air was used as the reference.

2.8. FTIR Analysis. FTIR spectroscopy was conducted on a PerkinElmer Spectrum 100 spectrometer with a Universal ATR accessory. IR spectra were collected from the samples within the range of 4000–650 cm⁻¹. Two areas were analyzed per sample, and 50 scans were recorded per spectrum. A background spectrum was recorded under the same experimental conditions and subtracted from each spectrum presented. An automatic baseline correction was done as postprocessing for all spectra.

2.9. XRD Analysis. The crystal structures of the nano-TiO₂ particles and the composite films were obtained using a D8 Advance Polycrystal X-ray diffractometer (Bruker, Germany) with a nickel-filtered Cu Kα radiation beam in the angular range of 6–70 °2θ at a voltage of 40 kV and a current of 40 mA.

2.10. Atomic Force Microscopy (AFM). AFM observations were carried out in the air on as-received sample surfaces using an Agilent S5000 AFM operated in contact mode. Measurements were carried out using standard triangular silicon nitride cantilevers (MSCF from Bruker). Topography, deflection, and friction data were collected simultaneously. The images were taken at 512 × 512 pixels resolution. Two areas of 50 × 50 μm were imaged per sample.

2.11. Inverse Gas Chromatography (IGC). IGC measurements were taken on an automated inverse gas chromatograph (Surface Measure Systems London, UK), equipped with both thermal conductivity (TCD) and flame ionization (FID) detectors. The specifications were according to Cordeiro et al.¹¹

2.12. Moisture Sorption Studies. Films were divided into tiny pieces and predried for 1 week at room temperature on silica in a vacuum. With saturated salt solutions of MgCl₂, MgSO₄·6H₂O, and Na₂SO₄·10H₂O, environments with constant relative humidities of 32, 53, and 95%, respectively, were created within the desiccators. These RH gradient values were selected because they correspond to most of the gradient observed within food materials or between packaged foods and surrounding media. The weights of the sample were checked until equilibrium was attained. The moisture absorption of the samples was calculated as follows:

\[
\text{Moisture absorption} = \left( \frac{w_1 - w_0}{w_0} \right) \times 100
\]

where wo and w1 were the weights of the sample before exposure to different relative humidities and after equilibrium, respectively. The average of three replicates of each sample was taken.¹²

2.13. Contact Angle. Contact angle measurement was done in a water contact angle instrument, SEO Phoenix 300, using software-8. Then, 6–7 μL drops of deionized water were placed on the surface of each film using a computer controlled microsyringe system under ambient conditions (temperature of 20 °C, relative humidity of 50%). The tangent angle formed between the film surface and the liquid drop was measured as the contact angle, and the drop image was captured using a CCD camera. Five different positions were tested for each film and the average taken.

2.14. Hemocompatibility Studies. To evaluate the blood compatibility of the film samples, a hemolysis assay and red blood cell aggregation studies were done. Appropriate protocols were followed for taking blood samples from willing patients. An experiment was carried out according to the procedure followed by Augustine et al.¹³

2.15. In Vitro Cytotoxicity Assay. The MTT assay was used for the assessment of cell proliferation (1929 fibroblasts) as reported in the earlier studies.¹⁴ Various SPI scaffold stocks (25 mg/10 mL) were prepared in DMEM. After incubation for 24 h, the suspension was centrifuged at 2000 rpm for 10 min, and the supernatant was filtered using a syringe filter and incubated with cells (ISO 10993-5, 2009) and further incubated for different timelines (24, 48, and 72 h).

2.16. In Vivo Experimental Design. In this study, there is a total of four experimental solvent cast SPI membranes: (1) SPI alone, (2) SPI blended with TiO₂ (1%), (3) SPI blended with CTE (7%), and (4) SPI blended with TiO₂ (1%) and CTE (7%). Before implantation, the SPI membranes were cut into 1 × 1 cm and sterilized using ethylene oxide (EtO). Animal experiments were designed and carried out as recommended in ISO 10993, part 6 (2016) with the approval of the Institutional Animal Ethics Committee of the host institution. Adult male Sprague–Dawley rats having a weight between 250 and 350 g were used for the study. The rats were provided with standard environmental controlled conditions of 25 ± 3 °C, a 12 h light-dark cycle, standard laboratory animal feed, and UV-sterile water. A total of 16 rats were randomized into four groups (n = 4) for subcutaneous implantation of SPI membranes, with (TiO₂/CTE) and without (blank).

On the day of surgery, rats were anesthetized by an intramuscular injection of 5% xylazine (10 mg/kg of the body weight) and 10% ketamine hydrochloride (80 mg/kg of the bodyweight). The hairs on the dorsum of the rats were shaved with a Phillips electric pet care trimmer, and the skin was cleaned using a 2% povidone–iodine solution. A linear incision of 8 mm length was made on the dorsum of the rat using a carbon steel surgical blade no. 15, and the full-thickness flap was elevated. All four sterile scaffolds were implanted and fixed using absorbable 3–0 catgut suture and were closed by giving interrupted sutures with number 3–0 black braided silk. In postoperative care, for 3 days, ceftriaxone (20 mg/kg) and meloxicam (0.5 mg/kg) were administered IM as an antibiotic and analgesic, respectively. The implanted scaffold samples were harvested by euthanizing four rats from each group in a CO₂ chamber at the first,
second, third, and fourth weeks. The connective tissue surrounding scaffolds was fixed in 10% formalin for histological evaluation. All sections were stained with Harris’s hematoxylin and eosin (H&E) stain for histological analysis. After H&E staining, sections were investigated histologically by observation under a polarized optical microscope (Leica DM 4500 P LED), and the images were captured. Sections were viewed and scored by a blinded pathologist using a four-point scoring system (0 absent, + mild, ++ moderate, and +++ severe) to determine the tissue reaction and membrane degradation.

2.1.7. Statistical Analysis. One-way analysis of variance (ANOVA) was performed with Duncan’s multiple range tests using the SPSS statistical analysis reference.

3. RESULTS AND DISCUSSION

3.1. Appearance and Color Parameters of SPI-TiO$_2$ Films. The photographs of (a) pure SPI film and (b) that containing 1% TiO$_2$ are shown in Figure 1. All of the films were smooth and homogeneous. The pure SPI film was see-through while the film containing 1% TiO$_2$ was translucent. It is observed that the transmittance decreases with an increasing concentration of TiO$_2$ in the protein matrix. This can be attributed to the great specific surface area and increased refractive index of the sonicated TiO$_2$ particles resulting in diffuse reflection of light from the film surface. Table 1 shows the color parameter values. L values show that lightness decreased with the increased addition of TiO$_2$ in the matrix. An increase in the value showed the color change to red/brown. The film’s hue changed from blue to yellow as the $b$ value grew.

3.2. SEM Analysis of TiO$_2$ and SPI-TiO$_2$ Films. Sonochemical synthesis of microparticles from macroparticles involves the application of ultrasound radiation (20 kHz-10 MHz) to form acoustic cavitation, i.e., the formation, growth, and impulsive collapse of bubbles that will generate hotspots, with high temperature ($5000-25,000$ K) and pressure (of more than $1000$ atm). This high local pressure and temperature, combined with rapid cooling, provides a one-of-a-kind method of synthesis of microparticles.

From the SEM analysis of the TiO$_2$ solution (Figure 2a and b), it is clear that after sonication for 1 h, the spherical particles of TiO$_2$ have been homogenized and the particle density has

---

Table 1. Color Parameters of SPI-TiO$_2$ Films

<table>
<thead>
<tr>
<th>samples</th>
<th>$L$</th>
<th>$a$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI</td>
<td>73.33 ± 1.53</td>
<td>3.93 ± 1.05</td>
<td>20.67 ± 2.08</td>
</tr>
<tr>
<td>0.5%TiO$_2$</td>
<td>59.06 ± 1.10</td>
<td>11.10 ± 1.15</td>
<td>38.33 ± 1.53</td>
</tr>
<tr>
<td>1%TiO$_2$</td>
<td>60.67 ± 3.21</td>
<td>10.33 ± 3.51</td>
<td>33.00 ± 2.64</td>
</tr>
</tbody>
</table>

$^a$Standard deviation ($\pm$) is given. Different small case letters within the same column indicate significant differences ($p < 0.05$).

---

Figure 1. Film appearance of SPI and SPI composite films.

Figure 2. SEM images of TiO$_2$ (a) before and (b) after sonication for 1 h. (Inset image in b shows the EDX spectra of TiO$_2$) SEM images of composite films (c) SPI, (d) SPI-0.5% TiO$_2$, (e) SPI-1% TiO$_2$. 

Scheme 2. Illustration Showing the Breakdown of Macroscale Titania to Microscale by Sonication and the Resultant Particles Uniformly Distributed in the SPI Matrix.
decreased when compared to a solution which has not been syndicated. The microstructure of nonsonicated TiO$_2$ samples showed the appearance of particle agglomerates and or large clusters, while after sonication a homogeneous dispersion of particles was achieved (Scheme 2). Here in Figure 2b, the size of the aggregates is reduced and individual particles or smaller clusters are formed. The average particle size was 180 to 205 nm. The inset image in b shows the EDX spectra of TiO$_2$, which contains only the peaks of Ti and O.

Figure 2c,d,e show the SEM images of the surface of the pure SPI and the TiO$_2$/SPI composite films. On examining the virgin SPI film surface, it seems to be even and devoid of any contamination. The physical appearance of the SPI film is thus supported by the morphology on the microscale. Thus, it can be simply said that the surface morphology of the pure SPI film was smooth and homogeneous. When moving to the composite films, a granular structure on the surface of the films appeared where TiO$_2$ was incorporated (Figure 2d,e). On close examination, the SEM images reveal rod-shaped TiO$_2$ microparticles that are homogeneously distributed in the matrix at 0.5% concentration and that tend to form small agglomerates as the concentration increases to 1%. Even then
the dispersion of the particles is nearly uniform, and they appear as particles in the matrix.

3.3. UV−Visible Transmittance Spectra. To evaluate the interaction between SPI-TiO₂ films and UV light radiation, UV−visible transmittance spectra of the composite films were taken. Figure 3A shows the UV transmittance spectra of the SPI/TiO₂ composite films with different TiO₂ concentrations. Due to the absorption of UV radiation by the aromatic amino acid groups of the proteins, the control SPI film has good UV blocking properties in the range 200−320 nm (UV-B and UV-C) but poor UV-blocking activity in the UV-A range (320−400 nm). The incorporation of TiO₂ into the SPI films caused a significant (p < 0.05) decline in the transmittance (T) in the UV-A, UV-B, and UV-C regions. More than 95% of UV light was prevented by even a 0.25% TiO₂ addition into the film. Thus, the incorporation of TiO₂ prevents free radical formation under UV light. It has also been reported by Li et al. that the addition of TiO₂ into whey protein isolate led to the decline of transmission in the visible, UVA, and UVB regions, and these declines showed a first-order exponential decay depending on TiO₂ concentration.

3.4. Fluorescence Spectroscopy. The fluorescence spectra of the composite films with different TiO₂ contents are shown in Figure 3B. The maximum emission wavelength of

Table 2. Surface Roughness, Surface Energy (Dispersive, Specific, and Total), Adsorption Potential Maxima (Aₘₐₓ) and Diffusion Coefficient (Dₛ) of the SPI-TiO₂ Samples

<table>
<thead>
<tr>
<th>sample</th>
<th>surface roughness</th>
<th>Yₛ(cm²/m²)</th>
<th>Yₛ(abm²)</th>
<th>Aₘₐₓ(kJ/mol)</th>
<th>Dₛ</th>
<th>Kₛ</th>
<th>Kₛ</th>
<th>Kₛ/Kₛ</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI</td>
<td>38.05</td>
<td>19.11</td>
<td>38.43</td>
<td>57.53</td>
<td>9.90</td>
<td>0.07</td>
<td>0.06</td>
<td>0.20</td>
</tr>
<tr>
<td>0.25%TiO₂</td>
<td>33.05</td>
<td>25.89</td>
<td>39.45</td>
<td>65.34</td>
<td>9.86</td>
<td>0.41</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>0.5%TiO₂</td>
<td>24.20</td>
<td>25.33</td>
<td>39.25</td>
<td>64.59</td>
<td>10.00</td>
<td>0.45</td>
<td>0.08</td>
<td>0.16</td>
</tr>
<tr>
<td>0.75%TiO₂</td>
<td>29.70</td>
<td>30.99</td>
<td>36.18</td>
<td>67.17</td>
<td>8.88</td>
<td>0.39</td>
<td>0.08</td>
<td>0.15</td>
</tr>
<tr>
<td>1%TiO₂</td>
<td>63.05</td>
<td>31.44</td>
<td>35.30</td>
<td>66.74</td>
<td>7.89</td>
<td>0.28</td>
<td>0.09</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Different small case letters within the same column indicate significant differences (p < 0.05).
which shows that TiO₂ addition, denatured state of SPI. When TiO₂ nm observed in our SPI films was different from the typical SPI emission maximum wavelength of 290 nm. The difference can be attributed to the effects of added glycerol and to the spatial conformation of the SPI chain, leading to improved functional properties in composite films. A better PL quench is also an indication of better dispersion of the filler in the matrix. Here, the highest quenching was observed in 0.5% TiO₂ concentration, whereas at high TiO₂ concentrations, self-assembled large TiO₂ accumulations formed could recuperate their crystallization ability.

3.5. X-ray Diffraction Analysis. Using the XRD pattern of pure TiO₂ (Figure 3C), a rutile characteristic structure was found (2θ = 25°, 36°, 41°, 44°, 54°, 56°, 64°, 70°). The XRD patterns of pure SPI and SPI with lower concentrations of TiO₂ (0.25%) show an amorphous nature. When 0.75% TiO₂ concentration is present in the film, a clear signal of diffracting peaks appears, indicating the presence of crystalline rutile TiO₂ in the SPI crystalline phase. At low TiO₂ addition, the coverage of protein on the surface of the TiO₂ particles reduced the crystallization characteristics of TiO₂, whereas at high TiO₂ concentrations, self-assembled large TiO₂ accumulations formed could recuperate their crystallization ability.

3.6. FTIR Spectroscopy. The molecular interactions, if any, between the TiO₂ and soy protein isolate in the composite films was studied using FTIR spectra. The spectral studies were done also to confirm whether the filler will have any adverse effect on the protein structure or not. In the FTIR spectrum of TiO₂ (Figure 4A), a single broad peak below 900 cm⁻¹ is attributed to the metal–oxygen (Ti–O) stretching band. The broad peaks at 3188–3428 cm⁻¹ and a small peak at 1631 cm⁻¹ is attributed to the OH stretching vibrations and bending vibrations of Ti–OH, respectively. All soy protein films exhibited three characteristic amide bands, amide I at 1628 cm⁻¹ attributed to the metal–oxygen (Ti–O) stretching band. The broad peaks at 3188–3428 cm⁻¹ and a small peak at 1631 cm⁻¹ is attributed to the OH stretching vibrations and bending vibrations of Ti–OH, respectively.

Table 3. Thickness, Solubility, Moisture Content, Water Contact Angle, UTS, and Elongation at Break of SPI-TiO₂ Composites

<table>
<thead>
<tr>
<th>samples</th>
<th>thickness (mm)</th>
<th>moisture content (%)</th>
<th>smooth</th>
<th>rough</th>
<th>ultimate tensile strength (MPa)</th>
<th>elongation at break (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPI</td>
<td>0.32 ± 0.011⁴</td>
<td>27.76 ± 1.20⁵</td>
<td>46.71 ± 2.09⁶</td>
<td>80.82 ± 5.45⁷</td>
<td>3.16 ± 0.22⁸</td>
<td>24.25 ± 1.99⁹</td>
</tr>
<tr>
<td>0.25%TiO₂</td>
<td>0.36 ± 0.002⁸</td>
<td>18.95 ± 0.61⁹</td>
<td>59.48 ± 2.44¹⁰</td>
<td>51.02 ± 0.64¹¹</td>
<td>3.47 ± 1.06¹²</td>
<td>120.83 ± 2.10¹³</td>
</tr>
<tr>
<td>0.5%TiO₂</td>
<td>0.38 ± 0.002⁸</td>
<td>10.10 ± 1.10¹⁴</td>
<td>38.23 ± 1.06¹⁵</td>
<td>48.63 ± 3.25¹⁶</td>
<td>4.58 ± 0.47¹⁷</td>
<td>95.31 ± 1.50¹⁸</td>
</tr>
<tr>
<td>0.75%TiO₂</td>
<td>0.39 ± 0.001⁵</td>
<td>17.38 ± 0.22¹⁹</td>
<td>39.03 ± 4.63¹⁰</td>
<td>48.85 ± 2.89¹¹</td>
<td>3.42 ± 1.25¹²</td>
<td>83.53 ± 2.28¹³</td>
</tr>
<tr>
<td>1%TiO₂</td>
<td>0.42 ± 0.001⁵</td>
<td>22.72 ± 1.12¹⁶</td>
<td>41.86 ± 3.04¹⁰</td>
<td>60.91 ± 1.03¹¹</td>
<td>2.69 ± 0.12¹²</td>
<td>65.47 ± 3.05¹³</td>
</tr>
</tbody>
</table>

“Values are determined as mean ± SD (n = 3). Lower case letters (a-e) in the same column indicate significant difference (p < 0.05).
cm⁻¹ (C=O stretching), amide II at 1538 cm⁻¹ (N-H bending), and amide III at 1136 cm⁻¹ (CN stretching), respectively. The addition of the TiO₂ into the SPI matrix caused the absorption band of the −OH group to move slightly to a higher wavelength (3264 cm⁻¹ to 3271 cm⁻¹). Furthermore, with the addition of 1% TiO₂, there occurs a small shift in the absorption intensity of amide I, amide II, and amide III bands, confirming the inclusion of TiO₂ in the SPI phase. As reported earlier, the addition of TiO₂ normally does not alter the basic protein structure of the SPI matrix. The small variations in the peak height and width are due to the presence of foreign material in the SPI matrix.

Oleyaei et al. have reported that the shift in the absorption peak of C−O bonds to the lower wavelength and the increase in the absorption peak intensity of CH stretching of the methylene group depicts the possibility of electrostatic interactions between the hydroxyl group of starch and TiO₂. The slight changes in the spectra obtained can also be attributed to such interactions.

3.7. AFM Analysis. AFM images were used to understand the surface roughness (quantitative) and morphology (qualitative) of composite films. Figure 5 shows the AFM
from the control and ranged from 38.43 to 35.30 mJ/m². For the soy samples, upon using TiO₂, it made the resulting composites more energetic (increased total surface energy up to 17%), resulting from the increase of the specific surface energy. The increment of the surface energy with the addition of TiO₂ could result from changes in the fiber conformation during the manufacturing process, which led to higher interaction with polar probes. Even though similar Y_s^d values (p < 0.05) were obtained between samples, the number and/or energy of the active sites could have changed. Thus, the heterogeneity profile was evaluated with the aim of accessing the active sites found at the surface (Figure 6A). Regarding the heterogeneity profile, some changes can be observed. At lower TiO₂ amounts (0.25% and 0.50%), the plot depicts a similar number (y axis) and energy (x axis) of the active sites. In the case of the samples with higher TiO₂ amounts, a significant decrease in the energy of the active sites is observed (A_max values decrease up to 20%), which agrees with the decrease in the Y_s^d value.

Even though similar Y_s^d values were obtained between samples, the number and/or energy of the active sites could have changed. Thus, the heterogeneity profile was evaluated with the aim of accessing the active sites found at the surface. At lower TiO₂ amounts (0.25% and 0.50%), the plot depicts a similar number (y axis) and energy (x axis) of the active sites. In the case of the samples with higher TiO₂ amounts, a significant decrease in the energy of the active sites is observed (A_max values decrease up to 20%), which agrees with the decrease in the Y_s^d value.

3.8. Polar Interactions. As seen through the analysis of the surface energy in the SPI samples, with the addition of TiO₂, the surface energy increased due to the increased polar interactions. This way, the Gibbs free energy of adsorption of different polar probes for the SPI-TiO₂ was assessed, which can be found in Figure 6B. With the incorporation of TiO₂, an increased interaction with the basic probes (ethyl acetate and THF) is observed, which translates into an increased acidic surface nature (higher K_a/K_b). Moreover, the interactions with dichloromethane (an acid probe) decreased, which further verifies the decrease of the surface basicity. It is most likely that variations in the polymeric chain orientation happened during the production of the membranes, which caused changes in the interactions with the polar probe molecules and, consequently, changes in the acid–base character. Due to the low amounts of TiO₂ used in the experiment, it is expected that it should not impact significantly the interactions with the probes under study.

3.8.3. Diffusion. The samples were subjected to diffusion analysis, using n-octane as the probe, and the parameter (D_p) can be found in Table 2. With the introduction of TiO₂ in SPI samples, the D_p is substantially increased. This indicates that the probe could cross much faster through the samples, indicating that a higher porosity was obtained in the presence of TiO₂ during the manufacture of the membranes. Increased surface porosity and diffusion values indicate the potential applicability of the film samples as a wound dressing material since it will permit the passage of oxygen to the wounded area, thus helping in maintaining optimum oxygen concentration to promote healing.

3.9. Film Thickness. The thickness of the final film is an important parameter which affects the migration rate of the liquid film-forming dispersion and hence is crucial for the mechanical and barrier properties of the films. Normally, film
thickness depends on the nature of film components, composition of the film-forming solution, preparation methods, drying conditions, etc. The thickness values of the SPI-TiO₂ composite are shown in Table 3. Film thickness was found to be in the range 0.032–0.04 mm. This indicated that the thickness values of SPI-TiO₂ composite films increased with an increase in the TiO₂ content. The results suggested that the alignment of SPI peptide chains in the film matrix was formed with a lower degree of compactness, when the increased content of TiO₂ was incorporated. The dispersed TiO₂ microparticles might have inserted themselves between SPI molecules of the film matrix, thereby hindering the interaction within the peptide chains.

3.10. Mechanical Properties. The stress–strain curves of SPI and SPI-TiO₂ films are shown in Figure 6C. A simultaneous enhancement in the tensile strength and elongation at break values was obtained with the addition of TiO₂ to the SPI matrix. As shown in the figure, the mechanical properties of the SPI films increased with the addition of TiO₂ up to 0.5% and then decreased. The film’s tensile strength is shown in Table 3. The tensile strength of the control SPI film was amplified and reached a maximum of 4.58 MPa with the TiO₂ concentration increased from 0 to 0.5% (p < 0.05). This shows that the addition of TiO₂ particles into the matrix solution resulted in strong interfacial interaction within the matrix network via the formation of covalent and hydrogen bonds. On further addition of TiO₂ from 0.75% to 1%, there was a significant decrease in the tensile strength, which may be due to the accumulation of TiO₂ particles in the matrix. This clearly shows that the uniform distribution of the microfiller in the matrix results in a specific and effective transfer of stress from the matrix to the filler, resulting in the increased strength of the film. The elongation at the break value was significantly (p < 0.05) increased with the addition of TiO₂. In fact, the addition of 0.25% TiO₂ was found to enhance the elongation at the break value by 120.83%. Balasubramanian et al. have also reported that the addition of TiO₂ increased the elongation at the break value of biopolymeric films.

3.11. Thermogravimetric Analysis. The thermal stability of the prepared SPI-TiO₂ composite films was analyzed by thermogravimetry. The thermogram (Figure 6D) revealed that all of the films exhibited similar degradation trends. A three-step degradation pattern was observed for all of the films. The first stage (70–100 °C) can be assigned to the loss of H₂O. The second stage between 100 and 250 °C is assigned to the loss of the plasticizer. The third stage occurs between 250 and 400 °C and is assigned to the breakdown of the protein backbone and other fillers. The total weight loss of SPI was 90.15%. The addition of TiO₂ was found to increase the thermal stability of SPI film. The temperature at which 50% weight loss in the SPI-1%TiO₂ composite film occurred was 322.66 °C, while for the control SPI film it was 309.16 °C. The decreased weight loss at lower temperatures on adding TiO₂ can be attributed to the high thermal stability of TiO₂ particles.

![Figure 10. Histology analysis of SPI membranes after in vivo implantation for showing the formation of blood vessels through implanted films (the circles represent the blood cells grown on the scaffold that represents angiogenesis).](https://doi.org/10.1021/acsbiomaterials.2c00548)
Oleyaei et al.\textsuperscript{27} have also reported an increase in the thermal stability of chitosan film on adding TiO\textsubscript{2} particles. The residual masses of SPI, SPI-0.25\%, SPI-0.5\%, SPI-0.75\%, and SPI-1\% TiO\textsubscript{2} films were 15.21\%, 17.23\%, 18.89\%, 19.15\%, and 19.17\%, respectively. This shows that the addition of TiO\textsubscript{2} can protect the film from losing weight by hindering the diffusion of volatile products of SPI, thus maintaining the integrity of films at high temperatures.

3.12. Moisture Content. Water sensitivity is one of the major problems of SPI-based films, which has limited their application. The pure SPI film had the highest moisture content. The moisture content of SPI/TiO\textsubscript{2} films was found to decrease with the increased addition of TiO\textsubscript{2} with 0.5\%TiO\textsubscript{2} addition giving a 64\% reduction (Table 3). This result was ascribed to TiO\textsubscript{2} getting trapped inside the SPI matrix, restricting the entry of water molecules, which resulted in decreased moisture content. In addition, the addition of micro-TiO\textsubscript{2} into the SPI matrix could cause strong hydrogen bonds to form between the two main components, which could prevent water diffusing through the films, thus reducing moisture content values. However, when more than 0.5 wt \% of TiO\textsubscript{2} was included, it aggregated to result in decreased effective filler dispersion in the polymer matrix and facilitated moisture permeation.

3.13. Wettability Properties. The water contact angle is a physical quantity which determines the surface wettability or hydrophilicity of the film. The surface wettability or hydrophilicity is an important parameter to be considered when selecting materials for skin scaffolds. It has a direct connection with cell proliferation and attachment. A low contact angle value indicates good surface wettability and hence hydrophilicity within the acceptable limit. According to the findings, the incorporation of TiO\textsubscript{2} in the SPI allows the cell growth. The SPI film surface is also suitable for the growth of the live cells. The small decrease in the viability rate after 72 h for the TiO\textsubscript{2} (1\%) films can be attributed to the roughness in the surface of the film as evident from the SEM images. The particle per unit space is more in the SPI-TiO\textsubscript{2} (1\%) composites, hence it slightly decreases the space for proper growth. However, the distribution of the microfiller is not so uniform that still there is enough space for the cells for healthy living and proliferation.\textsuperscript{29,30} The results show that the viability of the cells for all membranes prepared in this experiment was excellent on the whole, showing no cytotoxicity.

3.16. Histopathological Analysis after Subcutaneous Implantation in Adult Male Sprague–Dawley Rats. Usually, when a foreign body is implanted in vivo, it can result in an inflammatory response. A mild pro-inflammatory response facilitates tissue regeneration, while an unnecessary provocative response leads to organ impairment or abnormalities. Hence, it is important to study the in vivo performance of the material after implanting in animal bodies.\textsuperscript{31} Preclinical behavior of the prepared film membranes was studied using the subcutaneous implantation model. SPI and SPI-TiO\textsubscript{2} film membranes were subcutaneously implanted into male Sprague–Dawley rats for 4 weeks, and the inflammatory response, cell proliferation, and angiogenesis on the scaffolds at the end of the first, second, third, and fourth weeks of implantation were monitored (Table 4). The rats remained perfectly fine throughout the experiment without any mortality or abnormal behavior, suggesting the absence of toxicity on account of scaffold implantation. No sign of necrosis was observed in the neat SPI or SPI-TiO\textsubscript{2} scaffold groups throughout the study period. The healing of a scar or wound progresses through four stages in which there are platelets, neutrophils, fibroblasts, and epithelium. The fibroblast and...
angiogenesis represent the proliferation stage and are considered an indication of healing.\(22\) Superior angiogenic responses compared to the bare SPI samples were observed in SPI-0.5\%TiO\(_2\) and SPI-1\%TiO\(_2\). There was a significant difference in histological scores among the SPI membrane (blank) and SPI-TiO\(_2\) membranes in terms of angiogenesis, lymphocytes, polymorphonuclear leukocytes (PMNs), giant cells, plasma cells, and overall tissue inflammation response at different time points.

Interestingly, just after 1 week of implantation, angiogenesis was observed on the SPI-TiO\(_2\) membrane scaffolds (Figure 10). Only very few lymphocytes and giant cells were seen. PMNs were rare. Earlier studies\(15\) have proved that ultrasonication on TiO\(_2\) particles promoted neovascularization in rat models, which is attributed to the generation of reactive oxygen species (ROS) by TiO\(_2\) in biological systems. The metal oxide fillers have been proven to be effective in inducing cell proliferation and angiogenesis, and the mechanism has been explained by Augustine et al.\(34,35\) Reduction of inflammatory cells and induction of angiogenesis response in the SPI scaffold with TiO\(_2\) at the fourth week give evidence to the biocompatibility of the membrane.

\section*{CONCLUSION}

The foremost intentions of this work were to investigate (1) the effect of the sonication on the dispersion property of TiO\(_2\) and (2) the effect of addition of the sonicated TiO\(_2\) on the physicochemical properties of SPI films. It was found that sonication could enhance the dispersion of TiO\(_2\) in the matrix, and by adjusting the concentration of TiO\(_2\) microparticles, the properties of the TiO\(_2\)/SPI composite film could be tailored. The SPI-TiO\(_2\) films possessed a decreased transmittance in the UVA and visible regions and fluorescence quenching, which promoted its photocatalytic antibacterial effects. The mechanical strength and thermal stability of SPI films were enhanced in the presence of TiO\(_2\). The surface roughness, wettability, surface energy, and diffusion coefficient were significantly enhanced with the addition of TiO\(_2\). Biocompatibility studies showed 118\% L929 cell growth on SPI-1\%TiO\(_2\) scaffold. An in vivo implantation study demonstrated that TiO\(_2\) containing SPI membranes can enhance angiogenesis in the implantation site. Overall, the study indicated that SPI membrane scaffolds containing TiO\(_2\) can be used as a base material for the development of biodegradable scaffolds for wound healing applications. However, further investigations on teratogenicity, genotoxicity, and carcinogenicity need to be done to rule out any long-term adverse effects of the developed membrane scaffolds.

\section*{ASSOCIATED CONTENT}

\section*{Supporting Information}

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsbiomaterials.2c00548.

Water uptake studies (PDF)

\section*{AUTHOR INFORMATION}

\section*{Corresponding Author}

Lalı A. Pothen — Department of Chemistry, CMS College, M G University, Kottayam 686001 Kerala, India;
Phone: 9497785727; Email: lapothen@gmail.com

\section*{Authors}

- **Rekha Rose Koshy** — Postgraduate and Research Department of Chemistry, Bishop Moore College, University of Kerala, Mavelikara 690101 Kerala, India; Department of Chemistry, CMS College, M G University, Kottayam 686001 Kerala, India
- **Siji K. Mary** — Postgraduate and Research Department of Chemistry, Bishop Moore College, University of Kerala, Mavelikara 690101 Kerala, India; Department of Chemistry, CMS College, M G University, Kottayam 686001 Kerala, India
- **Arumina Regunadhyan** — Department of Chemistry, TKM College of Engineering, Kollam 691005 Kerala, India;
orcid.org/0000-0003-3658-3589
- **Yogesh Bharat Dalvi** — Pushpagiri Research Centre, Pushpagiri Institute of Medical Sciences, Tiruvalla, Kerala 689101, India
- **Lekshmi Kailas** — School of Physics and Astronomy, University of Leeds, LS2 9JT, London, United Kingdom
- **Nereida Cordeiro** — Faculty of Science and Engineering, University of Madeira, 9000-390 Funchal, Portugal
- **Sabu Thomas** — School of Energy Materials, Mahatma Gandhi University, Kottayam, Kerala 686560, India

Complete contact information is available at: https://pubs.acs.org/10.1021/acsbiomaterials.2c00548

Notes

The authors declare no competing financial interest.

\section*{REFERENCES}


---

**Recommended by ACS**

**Zonally Stratified Decalcified Bone Scaffold with Different Stiffness Modified by Fibrinogen for Osteochondral Regeneration of Knee Joint Defect**

Zhenyiin Chen, Yonggang Lv, et al.  
NOVEMBER 06, 2022  
*ACS BIOMATERIALS SCIENCE & ENGINEERING*  
[READ](https://doi.org/10.1021/acsbiomaterials.2c00548)

**Absorbable Electrosprun Poly-4-hydroxybutyrate Scaffolds as a Potential Solution for Pelvic Organ Prolapus Surgery**

Kim Verhoestert, Zeliha Guler, et al.  
OCTOBER 31, 2022  
*ACS APPLIED BIO MATERIALS*  
[READ](https://doi.org/10.1021/acsabm.2c00134)

**Spatially and Temporally Controllable BMP-2 and TGF-β, Double Release From Polycaprolactone Fiber Scaffolds via Chitosan-Based Polyelectrolyte Coatings**

Julius Sundermann, Heike Bunjes, et al.  
MAY 27, 2022  
*ACS BIOMATERIALS SCIENCE & ENGINEERING*  
[READ](https://doi.org/10.1021/acsabm.2c00088)

**Coordination of Osteoblastogenesis and Osteoclastogenesis by the Bone Marrow Mesenchymal Stem Cell-Derived Extracellular Matrix To Promote Bone Regeneration**

Huan Wu, Zhongbing Huang, et al.  
MAY 30, 2022  
*ACS APPLIED BIO MATERIALS*  
[READ](https://doi.org/10.1021/acsabm.2c00548)