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A Study of Hybrid Organic/Inorganic Hydrogel Films Based on in Situ-generated TiO_2 Nanoparticles and Methacrylated Gelatin

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Abstract: Methacrylated gelatin films with in situ-generated TiO_2 nanoparticles containing varying weight percentages of gelatin (0 %, 0.5 %, 1 %, 2 % and 4 %) were successfully prepared as novel biomaterials. ^{1}H-NMR spectroscopy confirmed their methacrylation with a 79 % degree of substitution. TiO_2 nanoparticles were uniformly distributed in the films with the average particle size increasing from 85 to 130 nm in proportion to an increase in TiO_2 concentration from 0.5 to 4 wt%. The water absorption of various gelatin methacrylamide/TiO_2 films was in the range of 471-758 %, which was enough to prevent wound beds from exudates accumulation. And in vitro degradation test in PBS showed that the three-dimensional structure of all samples basically remained unchanged although more than or nearly half the mass of specimens decreased after 4 weeks' degradation, and the pH levels of all sample solutions were maintained in an adequate range of 6.5-7.4 for cell and tissue growth during the whole process. The antibacterial activities of the films against *E. coli* and *S. aureus* were measured via a shake flask test and demonstrated good performance after the importation of TiO_2 nanoparticles. Cytotoxicity testing revealed that all films had no cytotoxicity and showed favorable adherence in the presence of L929 cells. The results suggest that hybrid hydrogel films hold potential for antibacterial wound dressing and tissue engineering scaffold applications.

Keywords: TiO_2 nanoparticles, Methacrylated gelatin, Antibacterial activity, Cytotoxicity

Introduction

Hydrogels belong to a physically or chemically cross-linked hydrophilic polymer network and can maintain a distinct three-dimensional structure while absorbing much water [1,2]. Due to their similarity to living tissues [3] and outstanding biocompatibility [4], hydrogels have received considerable attention from both tissue engineering [5] and pharmaceutical formulations [6] point of view. Nevertheless, the drawbacks of hydrogels, including low mechanical strength and difficulty for sterilisation, compromise their widespread biomedical application to a certain extent [7]. The development of superior hydrogels with improved mechanical performance and nontoxic biodegradability is therefore imperative.

Of the synthetic (PEO, PVA [4], etc.) and natural (chitosan, alginate [5], etc.) polymers used to form hydrogels, gelatin, which is derived from collagen, has been widely applied in wound dressing and tissue engineering [8,9], because it is completely degradable in vivo, has lower antigenicity than collagen and can be easily modified chemically with its large number of functional side groups [10]. Unfortunately, gelatin, in most cases, exhibits good solubility in aqueous environments and is not stable at body temperature because of its low melting point. Accordingly, the use of proper cross-linking agents to stabilize gelatin polymer is essential. Various chemical cross-linking methods that exploit biofunctional reagents, including glutaraldehyde [11], diisocyanates [12], proanthocyanidin [13], carbodiimides [14], genipin [15], polyepoxycompounds [16] and acyl azides [17], are currently available. Glutaraldehyde and formaldehyde are the most commonly used chemical regents for cross-linking gelatin, but they are both considered to be toxic to human cells [18,19] and have been reported to predispose biological tissue to calcification [20], owing to their chemical residual and biodegradation products. Therefore, the identification of cross-linking technology with less cytotoxicity and high efficiency is a hot research field. Since a gelatin methacrylamide (GelMA) hydrogel was first acquired by modifying gelatin with methacrylic anhydride (MA) and then cross-linking under UV irradiation [21], numerous studies on biomaterial applications, including porous scaffolds for tissue regeneration [22,23], cell-responsive microtissues [24] and interpenetrating polymeric networks [25,26] have been carried out. The findings indicate that methacrylated gelatin is a very promising biomaterial for wound healing and tissue engineering.

In addition, gelatin is susceptible to bacterial infection under adequate environmental conditions, which dramatically limits its medicinal applications. One potential approach to addressing this issue is to incorporate gelatin into antibacterial agents. TiO_2 nanoparticles with photocatalytic activity are commendable low-cost, biologically inert and non toxic antimicrobial inorganic compounds [27]. Various attempts to embed TiO_2 into polymers, such as polypropylene [28], ethylene-vinyl alcohol copolymer [29] and poly[2-(tert-butylamino) ethyl methacrylate-*co*-ethylene glycol dimethacrylate] [30], have been carried out, resulting in novel organic/inorganic

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nanocomposites with unprecedented performance against both Gram-positive and Gram-negative bacteria. Besides, in the light of their enormous surface areas, TiO_2 nanoparticles have a very active surface chemistry and hence tend to interact with the polymers in which they are dispersed, thus affecting the physical and chemical characteristics of nanocomposites. Xing [31] and our group [32] previously found that the water vapor transmission rates, tensile strength and elongation of the composites can be significantly modulated by the insertion of TiO_2 nanoparticles.

This study synthesized methacrylated gelatin polymer/TiO_2 nanoparticle composites (polymeric/TiO_2 nanoparticles) as biodegradable hydrogel films. The TiO_2 nanoparticles were synthesized via an in situ method in a polymeric matrix to maintain the stability of TiO_2 in the polymers. Hydrogel was cross-linked under UV irradiation to obtain water-resistant films. The nanocomposite films combined the advantages of gelatin and the functional properties of TiO_2, thus exhibiting great water absorption capability, biocompatibility and antibacterial efficiency.

Materials and Methods

Materials

Gelatin type B (isolated from bovine skin, 225 bloom) and Irgacure 2959 [purity: 98 %; 2-hydroxy-4'-(2-hy-droxyethoxy)-2-methyl-propiophe] were obtained from Sigma-Aldrich. MA was purchased from Adamas Reagent Co. Ltd (purity: 94 %; Switzerland). Tetrabutyl titanate and acetylacetone (acacH) were imported from Aldrich Chemical Co. Inc. (United States). All other reagents used were of analytical grade.

Synthesis of Methacrylated Gelatin

Fifteen grams of gelatin was dissolved in 150 m*l* phosphate of buffered saline (PBS; pH 7.4) and the mixture was mechanically stirred at 50 °C for 30 min. After the gelatin was completely dissolved, 2 m*l* of MA was added to the mixture with vigorous stirring. After 3 h of reaction, the product was dialyzed against distilled water with dialysis membranes (MWCO=14 kDa) at 40 °C for 48 h to remove free methacrylic acid and salts. The solution was then dried under vacuum.

Preparation of GelMA/TiO_2 Complex Solution

The titanium precursor was prepared by mixing different amounts of $Ti(OBu)_4$, acacH and anhydrous ethanol in a dry atmosphere. After ultrasonicated for 1 h, the precursor solution was added drop-wise to 20 wt% GelMA aqueous solution under tempestuous stirring at 30 °C, keeping the pH of the solution stable at 2.5-3 with hydrochloric acid.

Preparation of GelMA/TiO_2 Hydrogel Films

After 4 h of stirring, the complex solution was mixed with Irgacure 2959 and then deposited on a cast made of two transparent plastic plates separated by a 0.8 mm spacer and exposed to a LWUV lamp (6 W) for cross-linking for 30 min at 365 nm. This process converted the complex solution into a solid hydrogel. Eventually, the hydrogel was repeatedly washed with biodistilled water and then dried .

^{1}H-NMR

Pure gelatin and methacrylated gelatin specimens were dissolved in D_2O (purity: 99.9 %) at the concentration of 10 mg/m*l*. ^{1}H-NMR spectra were collected at room temperature and a frequency of 600 MHz using a Varian INOVA NMR spectrometer.

Scanning Electron Microscopy (SEM) Analysis

The morphology of the TiO_2 particles was investigated with a scanning electron microscope (JSM-5900LV; Japan). Cross sections were prepared by manually fracturing the hydrogel films and coating them with gold before observation.

Swelling Properties

The water sorption capacities and equilibrium water content of the hydrogel membranes were manipulated by immersing the known weight of the dried hydrogel plate (15 mm diameter and 0.8 mm thickness) in 50 m*l* of distilled water at 37 °C. After being soaked for a predetermined period, the plates were drawn from water, wiped off the excess surface water and reweighed. The swelling ratio and equilibrium water content of the hydrogel in aqueous medium were then calculated from as follows:

$$\text{Swelling Ratio (\%)} = \frac{W_s - W_0}{W_0} \times 100\%$$

$$\text{Equilibrium Water Content (\%)} = \frac{W_s - W_0}{W_s} \times 100\%$$

where W_s and W_0 are the final and initial sample weights, respectively. Data were reported as the mean±standard deviation (SD) of five parallel runs for each sample.

In vitro Degradation

The degradation of films was measured by fully soaking the dried specimens into the pH 7.4 PBS. The solid/fluid ratio was 20 mg/m*l*. The specimens were incubated in a polyvinyl tube at 37 °C and shaken with a horizontal shaker at 100 min^{-1}. The PBS was refreshed weekly. At predetermined time intervals, the specimens were taken out, washed with distilled water and dried under vacuum at 50 °C. Their degradation ability was determined according to the following formula:

$$\text{Degradation Rate (\%)} = \frac{W_0 - W_t}{W_0} \times 100\%$$

where W_t and W_0 are the final and initial dried sample

weights, respectively. Data were reported as the mean±SD of five parallel runs for each sample.

In addition, the pH of the PBS was also recorded by a pH meter (PHS-25, China) before being replaced.

Antibacterial Assessment

The antibacterial activities of the hydrogel were tested against Gram-negative *E. coli* (ATCC25922) and Gram-positive *S. aureus* (ATCC6538). Freeze-dried bacteria were resuscitated in a 40 °C water bath for 5 min. In total, 0.1 m*l* of suspension was added to 3 m*l* of Luria-Bertani media and the mixture was cultivated at 37 °C for 24 h. After the subculture of two generations, the bacterial suspension was diluted to a concentration of (1-3) $\times 10^5$ CFM/m*l* with sterile PBS. An equal amount test sample was placed in a 250 m*l* flask and mixed with 30 m*l* of the bacterial suspension [(1-3) $\times 10^5$ CFM/m*l*]. The solution was shaken on an agitation shaker at the speed of 300 rpm/min and 37 °C. After 0, 2 and 6 h, 0.1 m*l* of the suspension was collected from each flask and spread onto Luria-Bertani agar plates equally. The plates were incubated at 37 °C for 48 h, and counted on bacterial colonies.

Antibacterial activity was calculated according to the following equation:

$$\text{Antibacterial Activity (\%)} = \frac{A-B}{A} \times 100$$

where A (0 h) and B (2 or 6 h) are the bacterial colonies before and after shaking, respectively. Data were reported as the mean±SD of five parallel runs for each sample.

Cell Adhesion and Proliferation

Cell adhesion and proliferation on GelMA/TiO_2 films were assessed by L929 mouse fibroblast cells. The cells were cultured in Dulbecco's modified Eagle's medium, 10 % fetal bovine serum, 0.1 mg/m*l* penicillin and 0.1 mg/m*l* streptomycin. Cells were incubated at 37 °C and supplemented with 5 % CO_2 in a humidified chamber. The specimens were cut into small rounds with a diameter of 10 mm and thickness of 0.8 mm and then placed in a 24-well tissue-culture polystyrene plate. The L929 cells were then seeded onto the specimens or well surface and empty polystyrene wells (as control) at the density of 1×10^5 cells. After 1, 3 and 7 days of culture, the attached cells were fixed with 0.5 % glutaraldehyde, rinsed with PBS to remove the non-adherent cells and then dehydrated in a graded ethanol series (30 %, 50 %, 70 %, 80 %, 90 %, 95 % and 100 %). Specimens were treated with isoamyl acetate for ethanol replacement and dried using CO_2 critical point drying. The attached cells on the sample surface were observed by SEM. The viability of the cells in each well was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after the cells were seeded for 1, 3 and 7 days. Data were reported as the mean±SD of five parallel runs for each sample.

Results and Discussion

Preparation of Composites

The preparation process for GelMA/TiO_2 films is illustrated in Figure 1. GelMA was prepared using the reaction of the primary amine groups of gelatin with MA. The reaction occurs in alkaline environment. Under alkaline conditions, the -NH_2 of gelatin is nucleophilic and can react with the -COC- of MA to form an amide bond, instead of transforming into -NH_3^+ without nucleophilicity and blocking the reaction in acidic solution. Besides, methacrylic acid, as one of the resultant byproducts, will lower the pH of the solution when the reaction proceeds. Therefore, its level should be measured and mixed with a certain amount of sodium hydroxide solution every 30 min if necessary to keep the reaction mixture in an alkaline environment.

In addition, TiO_2 nanoparticles were generated from hydrolysis of a titanium precursor with an in situ method. This process precedes acid-catalyzed hydrolysis of $Ti(OBu)_4$. Hence, the pH of the solution should be maintained between 2.5 and 3 during the second step. Otherwise, tetrabutyl titanate could be hydrolysed rapidly and condensed to undesirable TiO_2 agglomerates once it encounters an aqueous environment or moisture. Consequently, we used acacH which can reduce the chemical reactivity of the nano-TiO_2 precursor as the chelating agent in this study. acacH could substitute the hydrolysable alkoxy groups of tetrabutyl titanate and produce $Ti(OC_4H_9)_3$-xacacx based on the following reaction [33]:

$$Ti(OC_4H_9)_4 + x\ \text{accacH} \rightarrow Ti(OC_4H_9)_{3-x}\ \text{acacx} + x\ C_4H_9(OH)$$

The less reactive Ti-acac groups acted as poison towards hydrolysis and condensation, thereby restraining the formation of TiO_2 agglomerates and precipitates in the GelMA matrix.

^{1}H-NMR Spectra

The ^{1}H-NMR spectra of gelatin and methacrylated gelatin are shown in Figure 2. For D_2O as a solvent, the signal at 4.7 ppm was attributed to the deuterated water, whereas the

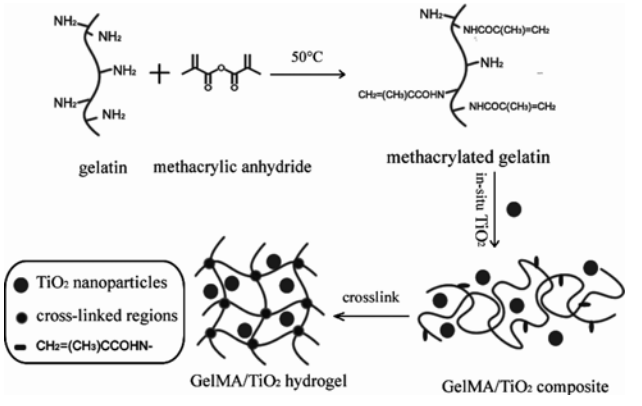

Figure 1. Schematic representation of preparation process of GelMA/TiO_2 films hydrogel.

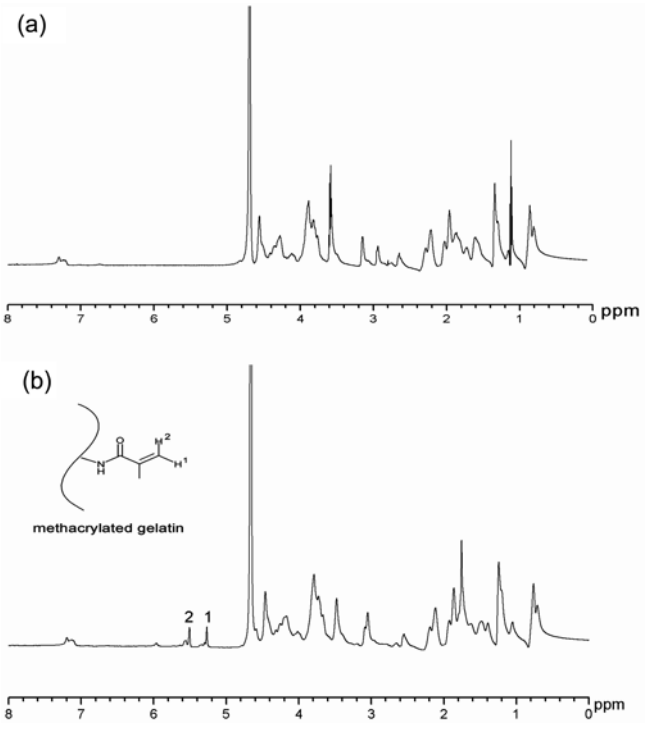

Figure 2. ^{1}H-NMR spectra of non-methacrylated (a) and methacrylated (b) gelatin.

aromatic residues triggered the chemical shifts between 7.1 and 7.3 ppm. The spectrum of methacrylated gelatin (Figure 2(b)) confirmed the immobilisation of the methacrylate double bonds at 5.2 and 5.5 ppm. Conveniently, the degree of substitution (DS), which is defined as the percentage of methacrylated e-amino groups of lysine and hydroxylysine residues to the total number of primary ε-amine groups of gelatin prior to methacrylation, can be calculated by comparing phenylalanine with methacrylate double bonds signals from the ^{1}H-NMR spectrum [34]. The DS depends on the molar reactant ratio of MA to gelatin amino residues and its value in this study was determined to be 79 %.

Morphology of the Specimens

SEM was used to characterise the morphology of TiO_2 nanoparticles embedded in GelMA. The morphologies of the blank and TiO_2-incorporated hydrogel films are illustrated in Figure 3. Panels (b-e) show that the TiO_2 particles were well separated and uniformly distributed in the GelMA matrix with an irregular shape on the whole, and this can be explained by the growth kinetics of TiO_2 nanoparticles in aqueous solution using tetrabutyl titanate as precursor [35]. On one hand, the average particle size was proportional to TiO_2 concentrition, ranging from 85 to 130 nm (not counted in the agglomerates; measured by SigmaScan Pro 5.0). On the other hand, with less than 1 wt% TiO_2, the TiO_2 nanoparticles scattered homogeneously in hydrogel films

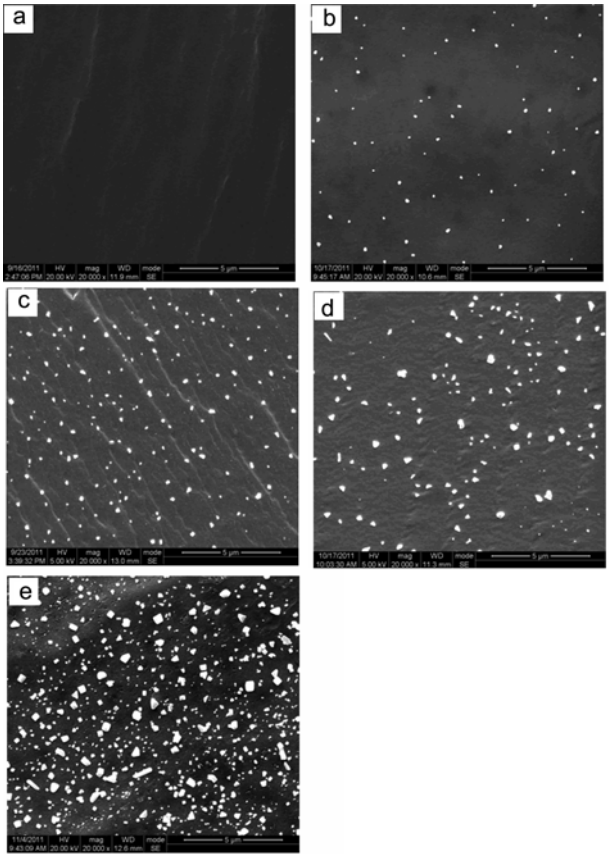

Figure 3. SEM images showing the cross section views of GelMA/TiO_2 films. (a) GelMA-0 (20,000×), (b) GelMA-0.5 (20,000×), (c) GelMA-1 (20,000×), (d) GelMA-2 (20,000×), and (e) GelMA-4 (20,000×).

instead of forming agglomerates. The dispersivity of TiO_2 in this study was better than that in previous research, in which TiO_2 nanoparticles were directly introduced into the matrix [31,36]. However, certain small nanoparticles would congregate to large agglomerates with an average size of approximately 400 nm when the concentration exceeded 1 wt%, because luxuriant TiO_2 nanoparticles had a propensity to reunite under an aqueous environment.

Water Absorption Ability

Water absorption ability is an important characteristic of hydrogel films, because materials with high water absorption could act similar to living tissues and absorb exudates or toxic components in medical applications. Figure 4 shows the swelling ratios of the hydrogel films, with all the specimens absorbing water promptly during the first hour and attenuated in the next 2 h. After 3 h of soaking, the equilibrium water uptake was reached. Similar to the pure gelatin that had a high water uptake of approximately 600 % in pH 7 aqueous solution [37], the water absorption and equilibrium water content of these specimens, as depicted in Figures 4 and 5,

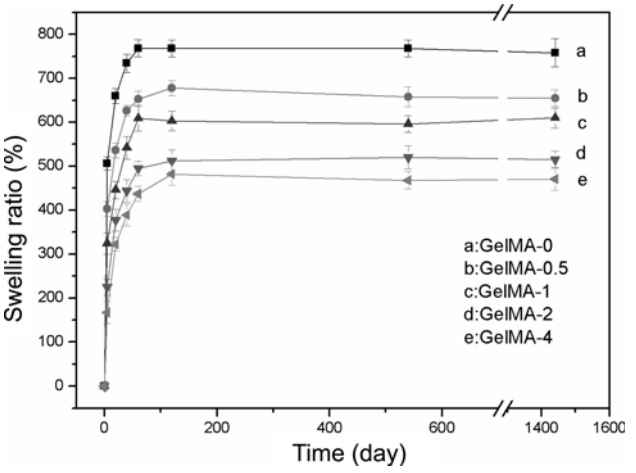

Figure 4. Swelling ratio properties of GelMA/TiO_2 hydrogel films. Error bars represent the SD of measurements performed on at least 5 specimens.

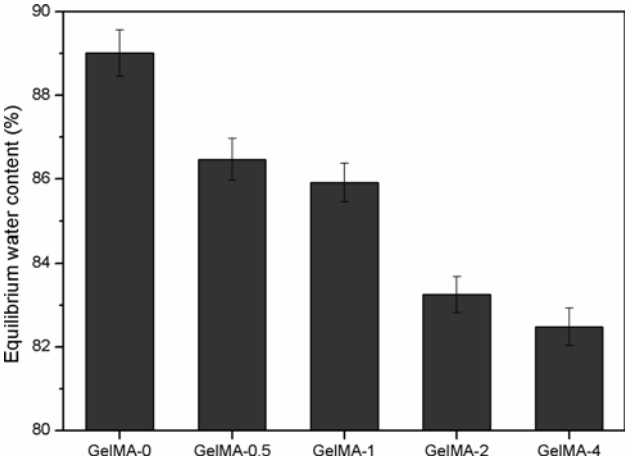

Figure 5. The equilibrium water contents of GelMA/TiO_2 hydrogel films. Error bars represent the SD of measurements performed on at least 5 specimens.

were in the ranges of 471-758 % and 82.5-89.1 % respectively, which also exhibited high water absorption capacity. These findings revealed that gelatin was still a kind of hydrophilic macromolecule [38]. Furthermore, the TiO_2 nanoparticles embedded into the gelatin networks could significantly reduce the water absorption capacity and equilibrium water content of hydrogel. These findings postulate that the COO-Ti bonds between TiO_2 nanoparticles and gelatin networks [39] can enhance organic/inorganic hydrogel films.

In vitro Degradation

Biodegradability is another significant property of hydrogel films, as materials with proper biodegradability can provide space for cell growth and rearrangement as well as tissue ingrowths when implanted into the body or during wound healing. In general, gelatin can be degraded into aminophenol

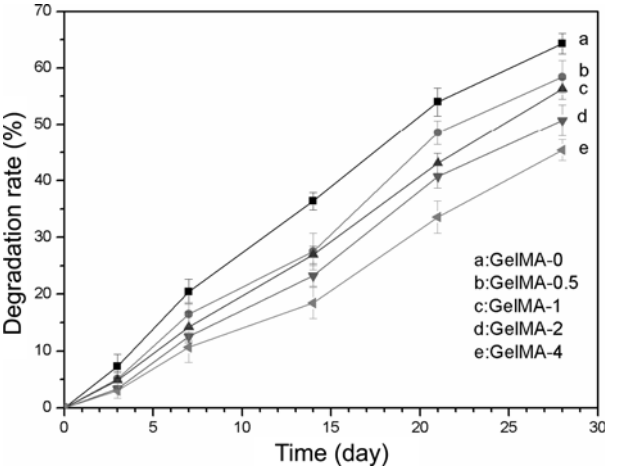

Figure 6. Degradation curve of GelMA/TiO_2 hydrogel films with time. Error bars represent the SD of measurements performed on at least 5 specimens.

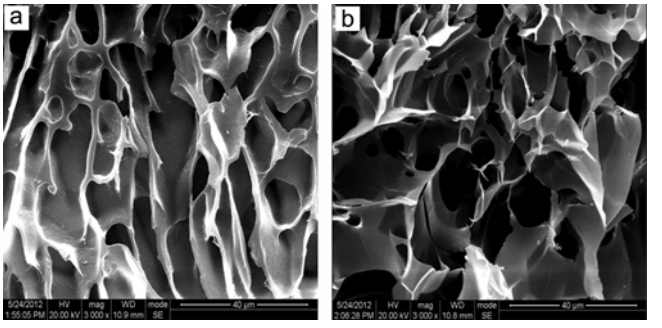

Figure 7. SEM macrographs of the GelMA-0 film in PBS before (a) and after (b) 4 weeks.

by virtue of hydrolysis and enzymolysis and then absorbed, resulting in metabolism or excretion from the body, thus avoiding the production of toxic byproducts, immunogenic responses and second operations. In this study, the degradation of GelMA/TiO_2 films was simulated in PBS at 37 °C. In most cases, unmodified gelatin would dissolve and lose its three-dimensional structure within a few hours in buffer solution at 37 °C. However, Figures 6 and 7 clearly show that gelatin cross-linked by MA could significantly enhance the stability of gelatin in PBS and basically maintain its three-dimensional structure in the solution for at least 28 days, which fulfills the fundamental requirement of some medical and tissue engineering applications.

Furthermore, all specimens dissolved by more than or nearly half after 4 weeks, thus allowing for cell and tissue ingrowth. Moreover, the incorporation of TiO_2 nanoparticles could enhance the hydrogel films that the degradation rate of gelatin decreased after inserting TiO_2 nanoparticles, as shown in Figure 6, which can be explained in two ways: (1) COO-Ti bonds can stiffen the hydrogel networks and (2) -COOH groups of gelatin with partial negative charges have an

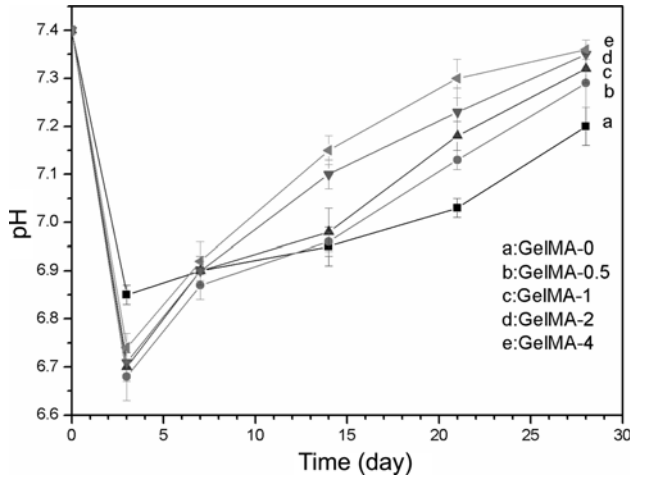

Figure 8. pH changes of PBS after GelMA/TiO_2 hydrogel films after soaking with time.

electrostatic interaction with the positive surface of the TiO_2 particles (-Ti^{+4}O-) under physiological conditions with degradation [40], both of which increased the stability of the solution and blocked further degradation. Besides, the pH of the PBS declined remarkably at first due to the incipient dissolution of unreacted acid anhydride and hydrochloric acid, and then increased slowly due to the exchange of the solution, as shown in Figure 8. Meanwhile, the pH levels of all sample solutions were maintained in the range of 6.5-7.4, basically guaranteeing the absence of adverse effects on cell and tissue growth.

Antibacterial Activity

Taking into account that sterilization is difficult but essential for hydrogels to prevent microorganism infection, their antibacterial activity is their most valuable property in the field of biomedical application. Here, the antibacterial activities of the hydrogels were tested against Gram-negative *E.coli* and Gram-positive *S.aureus* by a shake flask test for 2 and 6 h. As shown in Figures 9 and 10, neat GelMA did not exhibit any antibacterial ability against E.coli and S.aureus. On the contrary, it could provide nutrients, especially nitrogen sources, for bacterial proliferation. However, the death rates of *E. coli* and *S. aureus* increased from approximately 40 % to 80 % after 6 h whereas the concentration of TiO_2 increased from 0.5 % to 4 %. In addition, the death rate of bacteria after 6 h of immersion was almost two times higher than that after 2 h thereof using the same concentration of TiO_2. Therefore, both TiO_2 concentration and immersion time could significantly affect the antibacterial activities of the material. The mechanism of such activities was ascribed to the well-known photocatalytic behavior of TiO_2 nanoparticles. Reactive oxygen species generated from TiO_2 cause disorder in outer membrane of intact cells. In addition, after reactive oxygen species continue to damage the inner membrane of

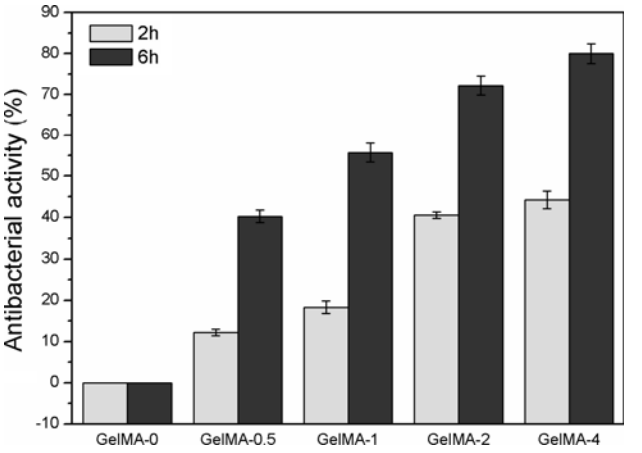

Figure 9. Antibacterial activity against *S. aureus.* Error bars represent the SD of measurements performed on at least 5 specimens.

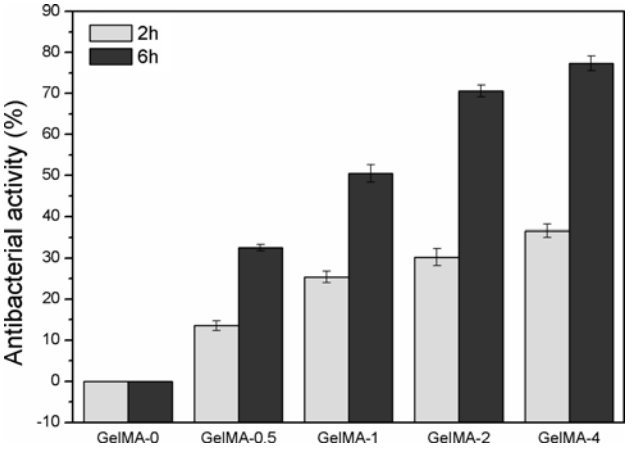

Figure 10. Antibacterial activity against *E.coli.* Error bars represent the SD of measurements performed on at least 5 specimens.

cells, the cells would die and degrade. Furthermore, free TiO_2 particles may also gain access to membrane-damaged cells, and the subsequent direct attack on the intracellular components can accelerate cell death [41].

Cytotoxicity

The primary issue in the development of biomedical materials is their cytotoxicity. An ideal antibacterial biomaterial should be able to decimate microorganisms without Cdamaging the host cells. Therefore, L929 cells were used in this study for the initial assessment of the cytotoxicity of GelMA/TiO_2 films. Figure 11 shows that the L929 fibroblast cells successfully attached to the films and that the number of cells on the surfaces of films increased with increasing incubation time, indicating that the films were conducive to cell attachment and proliferation. These demonstrated that gelatin cross-linked by MA and incorporated with TiO_2 nanoparticles did not have severe adverse effects on the cells. Figure 12 shows the MTT absorbance observed in cell proliferation experiments.

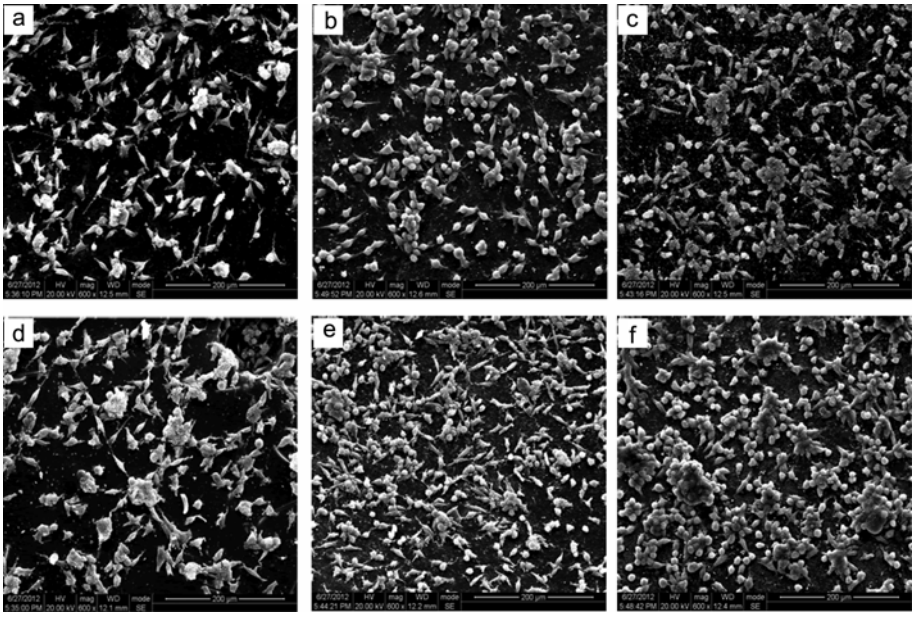

Figure 11. Morphology of L929 cells on the surface of GelMA films; (a) GelMA-0, after 1 day, (b) GelMA-0, after 3 day, (c) GelMA-0, after 7 days, (d) GelMA-3, after 1 days, (e) GelMA-3, after 3 days, and (f) GelMA-3, after 7 days.

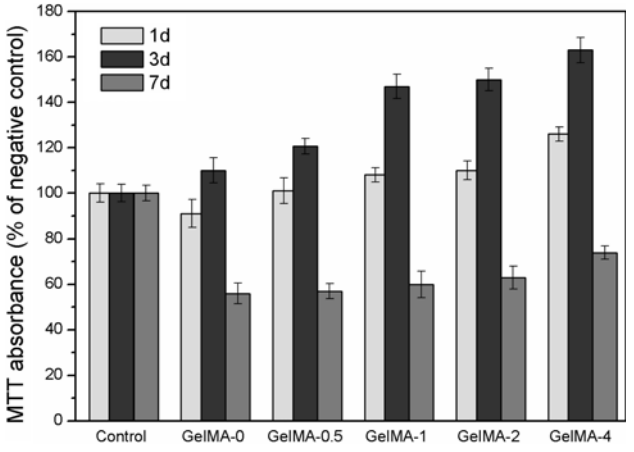

Figure 12. Cell proliferation on the surface of GelMA membranes. The absorbance was normalized with that of control at each time interval and the error bars represent the SD of measurements performed on at least 5 specimens.

All samples exhibited MTT absorption similar to that in the control after 1 day, but had higher rates (10-63 %) than the control as TiO_2 increased from 0 % to 4 % after 3 days. These results suggest that each increase the TiO_2 concentration was accompanied by a gradual but not great acceleration in the relative viability of the cells, which could be attributed to the probable that the films were likely to result in a rough surface after TiO_2 nanoparticles were incorporated and therefore exhibited better cell attachment. Interestingly, MTT absorbance dramatically decreased after 7 days, which is consistent with previous data [32]. These findings indicate that the addition of films could arrest the L929 cell cycle.

Conclusion

In summary, this study successfully prepared GelMA/TiO_2 nanoparticle films via an in situ method that allows for the uniform incorporation of TiO_2 nanoparticles into a polymeric GelMA matrix. ^{1}H-NMR and SEM confirmed methacrylation and TiO_2 incorporation, indicating that the hybrid organic and inorganic hydrogel films had a high DS and well-separated TiO_2 nanoparticles, respectively. The results of water absorption ability and degradation rate indicated that the films were able to shelter the wounds from exudates accumulation and provide enough space for cell growth during wound healing. Importantly, GelMA incorporated with TiO_2 nanoparticles exhibited exceptional antibacterial activities against *E.coli* and *S.aureus*, suggesting that the films could effectively prevent bacterial reproduction and protect wounds from bacterial invasion. In addition, the GelMA/TiO_2 films did not show detrimental effects on L929 cells. These unique properties of the GelMA/TiO_2 film make it a feasible and promising material for wound dressing and tissue engineering.

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