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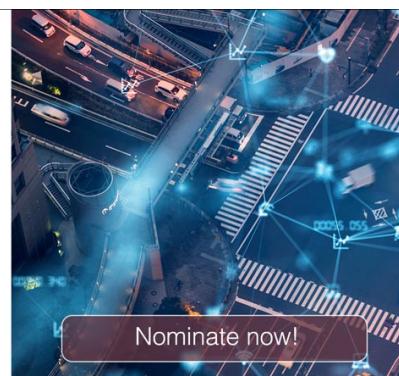


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Biomedical Materials



PAPER

Investigation of the influence of TiO₂ distribution on HA/TiO₂ composite wetting ability using the dispersant SDBS, high-temperature annealing, and ultrasonication

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Keywords: hydroxyapatite (HA)/TiO₂, surfactant SDBS, dispersion

Abstract

The use of composites such as hydroxyapatite (HA)/TiO₂ in bioapplications has attracted increasing attention in recent years. Herein, for the enhancement wetting ability and biocompatibility, the HA/TiO₂ composite was subjected to different treatments to improve nanoparticle (NP) distribution and surface energy with an aim of mitigating nanotoxicity concerns. The treatments included ultrasonication, high-temperature annealing, and addition of a dispersant and surfactant, sodium dodecylbenzenesulfonate (SDBS). Contact angle measurement tests revealed the effect of SDBS addition on the distribution of TiO₂ NPs on the HA surface: a decrease in the contact angle and, thus, an increase in the wetting ability of the HA/TiO₂ composite were observed. The combination of annealing and SDBS addition treatments allowed for guest TiO₂ particles to be uniformly distributed on the surface of the host HA particles, showing a rapid conversion from a hydrophobic to superhydrophilic property. *In vitro* investigation suggested that the cell viabilities of annealed HA/TiO₂, SDBS-added HA/TiO₂, and SDBS-added and annealed HA/TiO₂ reached 89.7%, 94.7%, and 95.8%, respectively, while those of HA and untreated HA/TiO₂ were 80.3% and 86.9%, respectively. The modified composites exhibited lower cytotoxicities than the unmodified systems (HA and HA/TiO₂). Furthermore, the cell adhesion behavior of the composites was confirmed through actin-4',6-Diamidino-2-phenylindole (DAPI) staining, which showed negligible changes in the cytoskeleton architecture of the cells. This study confirmed that a modified HA/TiO₂ composite has potential for bioapplications.

1. Introduction

In recent years, titanium dioxide nanoparticles (NPs) have been widely used in applications such as use as transparent screen-printable self-cleaning coatings and in water treatment. They have also been used as dye-sensitized solar cells, bioactive materials, and drug-delivery agents because of their high photocatalytic activity and chemical stability [1–4]. TiO₂-based nanomaterials have been coated on suitable substrates for different applications and have been synthesized by many methods including chemical vapor deposition [5, 6], sol–gel routes [7], atomic layer deposition [8, 9], and spin coating [10]. Among these, the spin-coating method using TiO₂ paste is the most effective

for the preparation of perovskite solar cells. TiO₂ nanomaterials have been widely used in biomedical applications, particularly to promote the growth of bone tissues [11].

The break-up and dispersion of NPs in a solution are essential to produce a TiO₂ suspension. Because of their large surface area and weak van der Waals forces, TiO₂ particles tend to show significant agglomeration [12]. Fortunately, agglomerated TiO₂ can be broken down into smaller agglomerates by applying mechanical force to disrupt the weak interactions. To enhance the dispersion of TiO₂ NPs, the number of NP clusters must be minimized. The dispersion of TiO₂ particles in water has been widely investigated [13, 14]. Although TiO₂ NPs

possess many remarkable advantages, their small size (<100 nm) allows the NPs to easily infiltrate into the human body, giving rise to nanotoxicity concerns [8, 15, 16]. Therefore, the effect of TiO₂ NP size on bone regeneration and material safety must be elucidated. Many physical dispersion methods to suppress agglomeration and stabilize dispersed TiO₂ NPs in solution have been reported, such as sonication, electrostatic treatment, and electrostatic stabilization [17]. One study reported the discovery of a stable dispersion of TiO₂ NPs on the surface of hydroxyapatite (HA), which is an inorganic constituent of human bones [18, 19]. The HA/TiO₂ composite is known to be an osteoconductive and biocompatible material because of its unique properties such as non-toxicity, corrosion resistance, fatigue durability, and biocompatibility [20–24]. TiO₂-coated HA has been found to significantly enhance new bone formation compared to HA [25]. The hydrophilicities of HA and HA/TiO₂ bone graft materials were increased via surface modification through ultraviolet photofunctionalization, leading to enhanced *de novo* osteogenesis [26]. This indicated that the nanoscale anodized Ti surface can play an important role in new bone formation during the healing reaction.

In this work, a multistep process to optimize the properties of the HA/TiO₂ composite was carried out by dispersing sodium dodecylbenzenesulfonate (SDBS) in an ethanol solution. SDBS has been widely used in various industries as a chemical surfactant because of its detergency stability, dispersibility, and moistening, foaming, and emulsifying properties [27]. The main objective of this study was to investigate the effects of ultrasonication, annealing, and SDBS addition treatments on the degree of distribution of TiO₂ NPs on the surfaces of host HA particles. Upon the addition of SDBS, the TiO₂ NPs could be maintained as a dispersion at a relatively stable and high concentration in ethanol. The wettability of the hydrophobic surface of the HA/TiO₂ composite was easily enhanced by using SDBS. The combination of annealing and surfactant addition treatments resulted in a rapid transition from a hydrophobic to superhydrophilic property, resulting in complete wetting. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay and actin-DAPI staining results indicated that the treated HA/TiO₂ composite was biocompatible and had the potential to mitigate nanotoxicity concerns arising from the use of biomedical materials. The use of the proposed composite, which has the benefits of facile preparation and cost-effectiveness, will facilitate the easy application of TiO₂ NPs in a liquid medium and will reduce the risk to human health arising from the use of nanoscale materials for bone regeneration [28].

2. Materials and methods

2.1. Materials

TiO₂ nanopowder (anatase, 99.99%, 25 nm) and SDBS (technical grade, purity >97.0%) were purchased from Sigma Aldrich. HA powder (Bongros) was purchased from Daewoong Pharmaceutical Co., Ltd, Seongnam, Korea. All chemicals were used directly as raw materials without further purification.

2.2. Sample preparation

TiO₂ NPs were dispersed in ethanol with or without the SDBS dispersant. TiO₂ and TiO₂-SDBS were dissolved in deionized water for 20 min under atmospheric pressure and then centrifuged twice at 4000 rpm for 40 min. Finally, the obtained powder was re-dispersed in 50 ml of ethanol. To prepare the HA/TiO₂ composite, 0.1 g of SDBS and 0.2 g of TiO₂ were mixed in 20 ml of deionized water at 200 rpm for 20 min. The obtained TiO₂-SDBS was mixed with 0.2 g of HA powder in 20 ml of ethanol and thoroughly stirred for 20 min at 200 rpm. The powder was collected by centrifugation, washed twice with ethanol, and dried overnight at 80 °C. The powder was subjected to ultrasonication and annealing at 450 °C (to maintain the TiO₂ phase). Five different composite samples were thus obtained and were designated as non-treated HA/TiO₂, HA/TiO₂-ultrasonication, HA/TiO₂-annealing, HA/TiO₂-SDBS, and HA/TiO₂-SDBS-annealing.

2.3. Characterization

2.3.1. Dynamic light scattering (DLS)

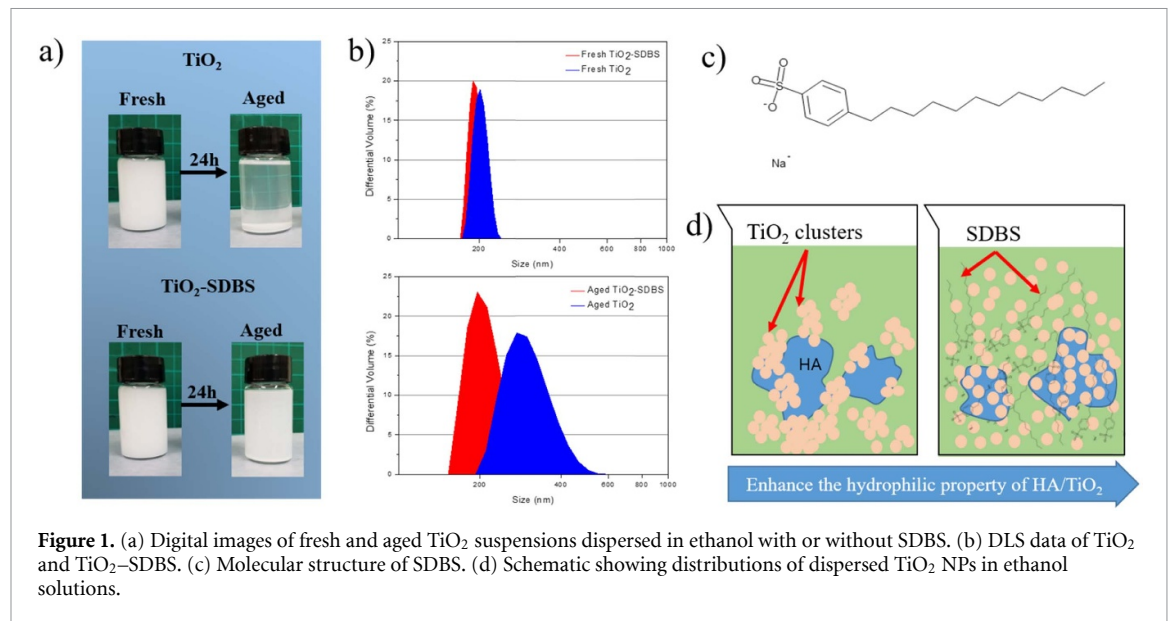
The hydrodynamic sizes of the TiO₂ and modified TiO₂ NPs in diluted water were determined using an OTSUKA Electronics ELSZ-2000Z system (Otsuka Electronics Co. Ltd, Japan) and a DLS technique. Zeta potential measurements of the TiO₂ NPs were performed using a zeta potential analyzer (ELSZ-2000Z, Otsuka Electronics Co. Ltd, Japan). Fresh samples were measured immediately after stirring, while aged samples were measured after one day of storage.

2.3.2. X-ray diffraction (XRD) analysis

The XRD analysis of the HA/TiO₂ and treated HA/TiO₂ composites (Rigaku, D/MAX 2200, Japan) was conducted with Cu K α radiation at 2 θ of 15°–60° at angular steps of 2° min⁻¹.

2.3.3. Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra were measured using a Nicolet 6700 spectrometer. The powder was mixed with spectroscopic-grade potassium bromide (KBr) in a 0.5 wt% mixture. The mixed powder was pressed into pellets at 35 MPa for 10 min. The spectrum of the



transparent pellets was recorded at a wavenumber range of $4000\text{--}400\text{ cm}^{-1}$ with a resolution of 4 cm^{-1} .

2.3.4. Water contact angle measurement

The hydrophilicity of the particles was evaluated through water contact angle measurement. The wetting was determined by measuring the contact angle of samples formed using a pressing die (diameter of 12 mm) at 200 bar for 10 min. The surface wettability of each product was evaluated using a contact angle meter (G10/DSA10 from Kruss AG). The surface free energy was calculated on the basis of Young's equation:

$$\sigma_s = \sigma_{sl} + \sigma_l \cos \theta,$$

where σ_s is the surface energy of the solid, σ_{sl} is the interfacial tension between the liquid and solid, σ_l is the surface tension of the liquid, and θ is the contact angle of the liquid on the solid.

2.3.5. Scanning electron microscopy (SEM)

The surface morphology of each composite was observed through SEM (Hitachi-S4700, Jeol Ltd, Tokyo, Japan) after coating it with a thin Pt layer under vacuum. The elemental composition was determined by combining SEM and energy dispersive x-ray (EDX) spectroscopy (SEM-EDX), wherein selected areas of the composites were analyzed.

2.4. In vitro cell culture experiments

2.4.1. MTT assay

An MTT assay was used to evaluate the cell viability. HeLa cells (ATCC # CCL-2) were seeded in 96-well plates (2×10^4 cells/well) for the proliferation assay. After being treated with each type of sample (0.1 mg ml^{-1}) at several time periods, the cells

were incubated with a cell proliferation kit (Sigma Aldrich) for 1 h at 37°C in the dark. Then, a microplate reader (Synergy H1, BioTek, VT, USA) was used to measure the absorbance intensities at 450 nm. In the concentration-dependent MTT assays, the HeLa cells were treated with increasing concentrations ($0.1, 0.5, 1$, and 2 mg ml^{-1}) of each composite sample for 4 h, followed by the same steps as the above experiments.

2.4.2. Actin-DAPI staining

The HeLa cells were treated with the samples (0.1 mg ml^{-1}) for 4 h. Next, they were sequentially treated with anti- β -actin antibody (Sigma Aldrich, USA), Alexa Fluor® 488-Goat anti-mouse IgG H&L (Abcam, USA), and DAPI (Sigma Aldrich, USA). Next, the cells were observed with a fluorescence microscope (Olympus, CKX53, Tokyo, Japan).

3. Results and discussion

3.1. Effect of SDBS on dispersion ability of TiO_2 suspension

The key to the efficient synthesis of homogeneous HA/ TiO_2 composites using an *in situ* method was to find a solution that would produce uniformly dispersed guest particles (TiO_2) on the surfaces of the host HA particles. TiO_2 is known to have poor dispersion ability because of its high surface area and intrinsic hydrophilicity [29, 30]. To overcome these limitations, SDBS (dispersant) was selected to prepare a dispersion of TiO_2 in ethanol that was stable for at least 24 h. Figure 1(a) shows the TiO_2 NP (P25) suspension containing the dispersant after storage for 24 h at ambient temperature (23°C – 25°C). The dispersion maintained a cloudy and opaque state. In contrast, a suspension of the high-surface-area TiO_2 NPs without SDBS was completely agglomerated after

Table 1. Particle sizes of TiO₂ dispersions determined by DLS and their corresponding zeta-potential values.

Samples	Particle size (nm)	Zeta potential (mV)
Fresh TiO ₂	200.5	−22.68
Fresh TiO ₂ –SDBS	192.9	−66.19
Aged TiO ₂	302.6	—
Aged TiO ₂ –SDBS	207.0	—

24 h of standing time. This phenomenon was caused by the interparticle van der Waals interaction, which commonly occurs in metal oxide particles.

DLS measurement was conducted to analyze the effect of SDBS on TiO₂ NP distribution in ethanol (figure 1(b)). For a fresh dispersion, the particle sizes of TiO₂ and TiO₂–SDBS remained unchanged at approximately 200 nm, while the particle size increased to 207 nm (TiO₂–SDBS) and 302 nm (TiO₂) after aging (table 1). The zeta potentials of the fresh TiO₂ and TiO₂–SDBS dispersions were found to be −22.68 and −66.19 mV, respectively. The electrostatic potentials of the dispersions revealed the ability of SDBS to cause the TiO₂ NPs to repel each other, which limited aggregation in the solution. Figure 1(c) depicts the chemical structure of SDBS. Figure 1(d) illustrates the distribution of the TiO₂ NPs in the dispersions with or without SDBS. The alkyl chain of SDBS weakened the interactions between neighboring TiO₂ NPs, which stabilized the homogeneous dispersion of the NPs. Therefore, SDBS could be applied as a dispersant to prepare homogeneous HA/TiO₂ composites with long-term stability.

3.2. Structure of HA/TiO₂ composites

The compositions and crystalline phases of the treated and non-treated composites were examined through XRD analysis. The sharp peaks suggested that all synthesized products were crystallized. Figure 2(a) shows the XRD patterns of the HA, treated and non-treated composites. The peaks observed at $2\theta = 25.33^\circ$, 37.86° , and 54.12° corresponding to the orientations along the (101), (001), and (105) planes, respectively, confirmed the formation of anatase-phase TiO₂. Prominent peaks were observed at 31.79° , 32.19° , and 32.93° corresponding to orientations along the (211), (112), and (300) planes, respectively, which confirmed the presence of HA. All spectra clearly indicate the presence of HA and anatase TiO₂, confirming that no reaction occurred between them. After the treatments, there was no observed change in the HA or TiO₂ peak positions or formation of new peaks upon SDBS addition. This indicated that the addition of the anionic dispersant SDBS, high-temperature annealing, and ultrasonication did not change the HA/TiO₂ crystal structure.

Figure 2(b) shows the FTIR spectra of the treated and non-treated composites. The FTIR spectra show similar absorption bands in three regions, corresponding to the vibrations of PO₄^{3−}, OH[−], and CO₃^{2−}.

Based on the experimentally observed band frequencies, the characteristic bands associated with the PO₄^{3−}, OH[−], and CO₃^{2−} vibrations appear at 964 (ν_1 band), 3560, and 1200–1800 cm^{−1}, respectively [31, 32]. The FTIR spectra of the composites clearly illustrate the presence of negligible amount of any TiO₂ phase in the region of 400–800 cm^{−1}. The close similarity between the FTIR spectra of the treated and non-treated composites indicates that the absence of organic peaks arising from the presence of SDBS in the composite samples. The incorporation of the guest TiO₂ NPs onto the HA host surfaces was further confirmed by XRD analysis.

3.3. Water contact angle measurement

To understand the effect of the treatments on the wetting property of the HA/TiO₂ composite, the contact angles of the composite samples were measured, as shown in figure 3(a). In the case of the non-treated HA/TiO₂, the water contact angle showed a half-spherical shape with a value of approximately $77.5 \pm 3^\circ$ (figure 3(b)). After ultrasonication and annealing at 450 °C (figures 3(c) and (d), respectively), the surface wetting property changed, showing a decrease in the contact angle from the original value to $53.7 \pm 3^\circ$ and $24.5 \pm 3^\circ$, respectively. The annealing temperature significantly affected the surface energy, resulting in a significant reduction of contact angle. Importantly, the SDBS-added composite showed a remarkably lower contact angle ($19.4 \pm 3^\circ$; figure 3(e)) than the non-treated HA/TiO₂ composite, which was attributed to the increase in surface energy upon SDBS addition. Finally, when the annealing and SDBS addition treatments were combined, the HA/TiO₂ composite exhibited a water contact angle of 0° (figure 3(f)), which verifies the applicability of the treated HA/TiO₂ composite in biochemical systems.

Figure 4 shows the decrease in the water contact angle and increase in the surface energy of the different HA/TiO₂ composites. The surface free energy of the HA/TiO₂ composite was 15.75 mN m^{−1}, whereas the surface free energy values of the modified HA/TiO₂ composites were higher (43.3–72.8 mN m^{−1}). This was in good agreement with previously reported results [33]. The ultrasonication treatment facilitates the conversion from a hydrophobic to hydrophilic property, which is governed by factors such as surface energy, affinity to water, and distribution of exposed crystal faces [34].

3.4. SEM–EDX

The surface morphology of HA/TiO₂–SDBS was analyzed through SEM and EDX measurement to better understand the conversion of the surface from hydrophobic to hydrophilic. The morphology of the HA particles was confirmed by the SEM image showing HA particles without TiO₂ NPs on their surfaces. Figure 5 shows the SEM

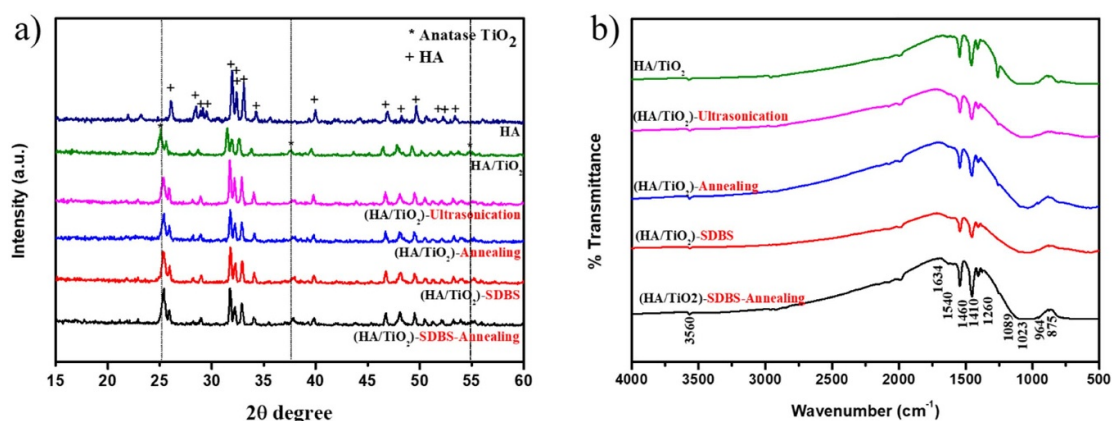


Figure 2. (a) XRD patterns of HA/TiO₂ after different treatments. (b) FTIR spectra of HA/TiO₂ after different treatments.

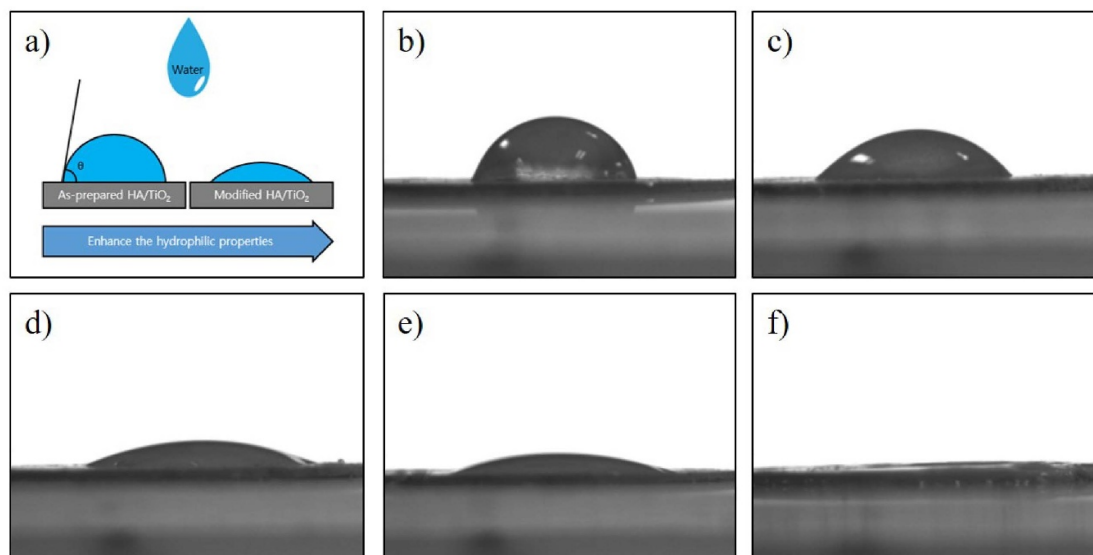


Figure 3. (a) Determination of hydrophilicity by water contact angle measurement. Contact angle images of (b) HA/TiO₂, (c) HA/TiO₂-ultrasonication, (d) HA/TiO₂-annealing, (e) HA/TiO₂-SDBS, and (f) HA/TiO₂-SDBS-annealing.

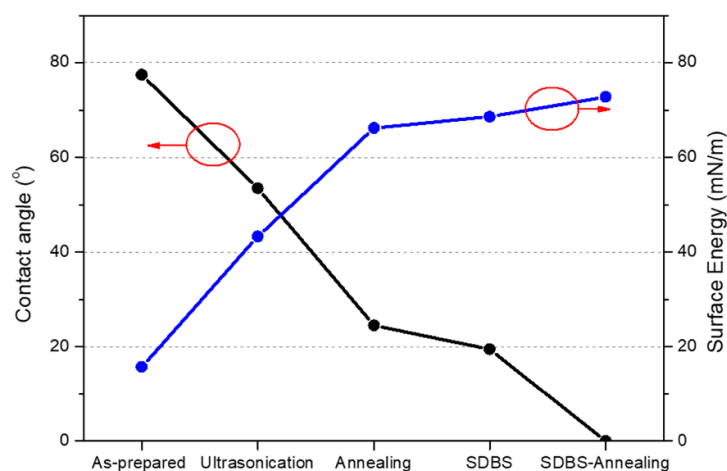
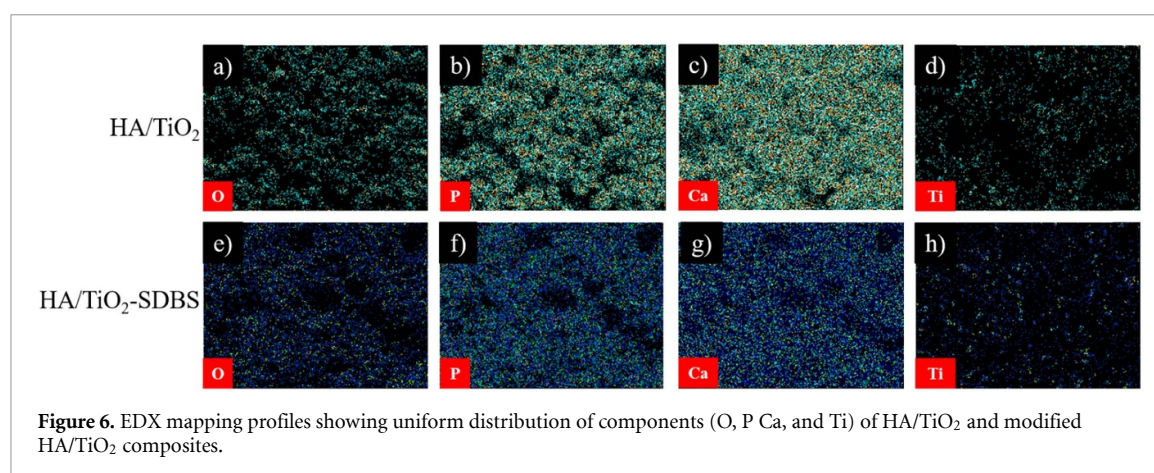
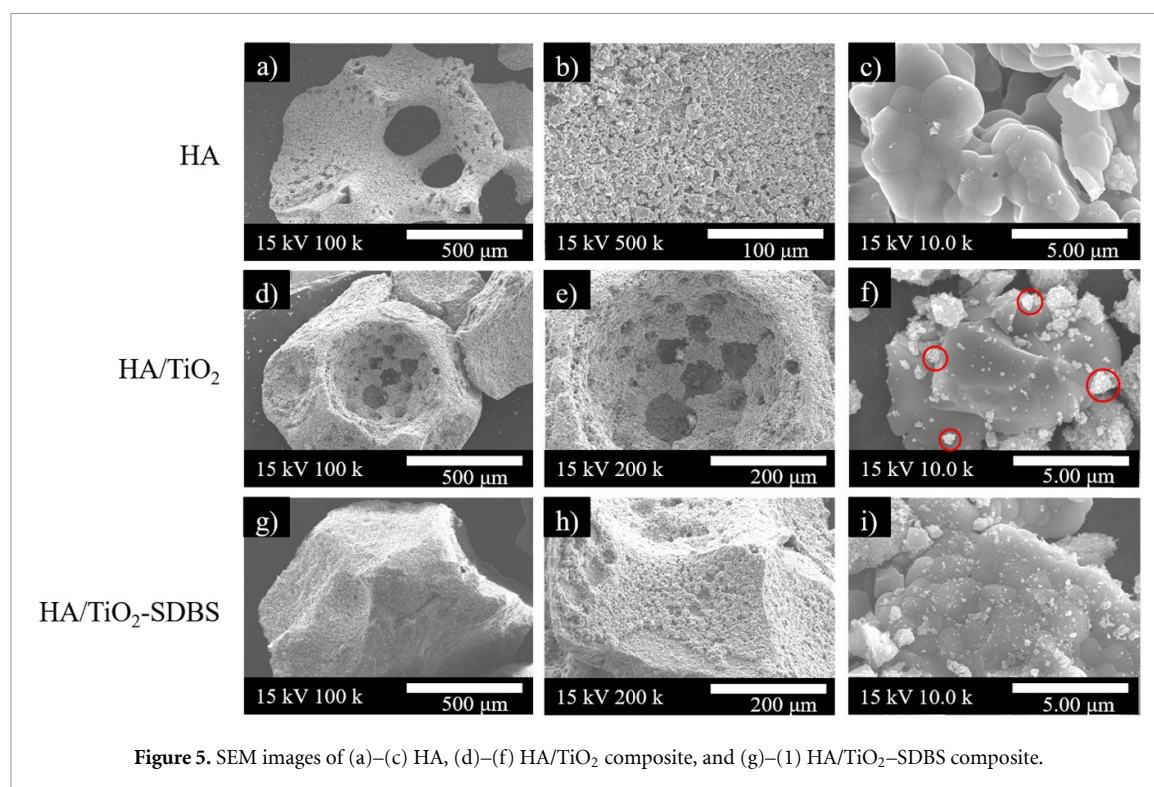


Figure 4. Contact angle values (blue line) and surface energy values (black line) of HA/TiO₂ and modified HA/TiO₂ composite.



images of the HA/TiO₂ and modified HA/TiO₂ composites, which were used to assess the quality of the composite in terms of TiO₂ dispersion on the HA surface. It can be observed that the TiO₂ NPs, which were easily embedded into surface of the HA with mechanical force because of their relatively small size, were gradually attached to the HA surface. TiO₂ clusters were clearly observed on the surface of the non-treated HA/TiO₂ composite, while HA/TiO₂–SDBS showed uniform surface distribution of TiO₂ on the HA surface.

No obvious TiO₂ clusters were observed in the surface microstructure of treated composites. A more detailed analysis of the chemical composition of the surface of the HA/TiO₂ and HA/TiO₂–SDBS by elemental mapping through EDX showed the presence of Ca, P, and Ti (figure 6). The Ti mapping profile

of the modified HA/TiO₂ composite showed uniform distribution in the examined area, unlike that of the HA/TiO₂ composite. It was confirmed that the addition of SDBS, which served as a dispersant, facilitated the homogeneous distribution of TiO₂ on the host surface, resulting in rapid conversion from the hydrophobic to hydrophilic property.

3.5. *In vitro* studies

3.5.1. MTT cell proliferation assay

The results of the MTT cell proliferation assay clearly demonstrated that the current HA/TiO₂-based system did not show any significant toxicity to the employed HeLa cell culture (figure 7(a)) after up to three days of incubation. Importantly, HA/TiO₂-annealing, HA/TiO₂–SDBS, and HA/TiO₂–SDBS-annealing showed higher cell viabilities than the unmodified samples (HA and HA/TiO₂), indicating

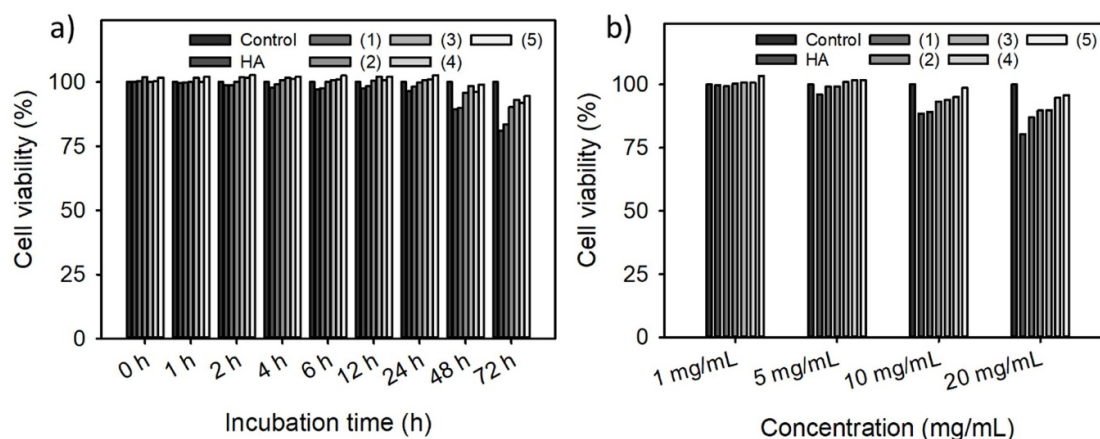


Figure 7. Cell viabilities of HeLa cells (a) after incubation with 0.1 mg ml^{-1} solutions of HA, HA/TiO₂ (1), HA/TiO₂-ultrasonication (2), HA/TiO₂-annealing (3), HA/TiO₂-SDBS (4), and HA/TiO₂-SDBS-annealing (5) and (b) with different sample concentrations after 4 h of incubation.

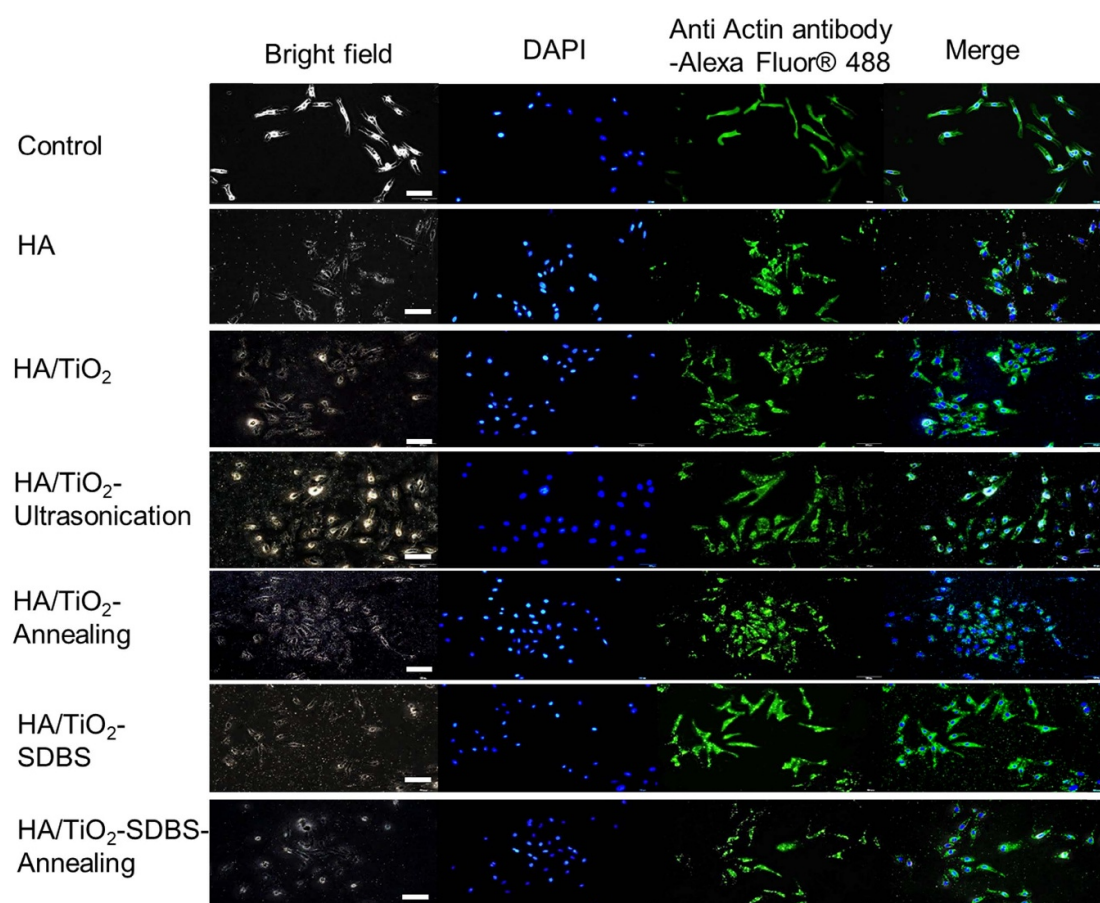


Figure 8. Fluorescent images of HeLa cells after 4 h of incubation with 0.1 mg ml^{-1} solutions of HA, HA/TiO₂, HA/TiO₂-ultrasonication, HA/TiO₂-annealing, HA/TiO₂-SDBS, and HA/TiO₂-SDBS-annealing with sequential treatment of anti- β -actin antibody, Alexa Fluor® 488-Goat anti-mouse IgG H&L, and DAPI. Scale bars: 100 μm .

that the treatments could mitigate the nanotoxicity concerns arising upon the biomedical application of the composites. In concentration-dependent toxicity evaluations, HA/TiO₂-annealing, HA/TiO₂-SDBS, and HA/TiO₂-SDBS-annealing exhibited lower cytotoxicity values than the unmodified systems (HA

and HA/TiO₂) (figure 7(b)). The viabilities of the cells treated with 2 mg ml^{-1} of HA/TiO₂-annealing, HA/TiO₂-SDBS, and HA/TiO₂-SDBS-annealing reached 89.7%, 94.7%, and 95.8%, respectively, while those of HA and HA/TiO₂ were 80.3% and 86.9%, respectively.

3.5.2. Actin-DAPI staining

The experimental results showed that there were negligible changes in the cytoskeleton architecture of the cells that had been incubated with the treated composites compared to that with the non-treated samples (figure 8). This confirmed that the employed samples did not affect the cytoskeleton architecture of the cells.

4. Conclusions

In this study, HA/TiO₂ composites were subjected to ultrasonication, high-temperature annealing, and SDBS addition treatments. The addition of SDBS, which served as a dispersant, improved the dispersion and stability of the TiO₂ NPs in the ethanol solution, contributing to a uniform dispersion of TiO₂ on the HA host surface. The surface hydrophilicity of the modified HA/TiO₂ composites increased with the enhancement of surface free energy compared with that of the unmodified composites. These changes not only enhanced the properties of the composite but also maintained the chemical composition and scaffold structure of HA, which is necessary for bioapplications. The MTT and cell adhesion assays showed that the ultrasonication, annealing, and SDBS addition treatments could reduce cytotoxicity and induced a negligible change in the cytoskeleton architecture of the cells. Therefore, the modified HA/TiO₂ composite is expected to show excellent bioactivity and fast bone growth and can be used as a biocompatible materials.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

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