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ORIGINAL ARTICLE

Surface Modification of Titanium Using BSA-Loaded Chitosan and Chitosan/Gelatin Polymers

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Abstract The integration of titanium-based implants with the surrounding bone tissue needs to be improved to increase their service life. This work presents a surface modification technique to increase the osteointegration of titanium implants. The studies were conducted in three main steps: (i) surface modification of titanium using bovine serum albumin (BSA)-loaded chitosan and chitosan/gelatin polymers, (ii) studies of BSA release from these surfaces, (iii) effect of porosity and polymer composition on osteoblast cell proliferation. TiO_2 and COOH groups were formed on titanium surfaces. Then, chitosan and chitosan (C)/gelatin (G) with various ratios (G:C = 0.5:1, 1:1, 1:0.5) were mixed with BSA and fixed to the surface via carbodiimide chemistry (EDC/NHS). After the immobilization process, samples were exposed to either air or freeze-drying. Characterization studies were conducted using Fourier transform-infrared spectroscopy and scanning electron microscopy. Finally, BSA release studies in phosphate-buffered saline (0.1 M, 37 °C) and cell (osteoblast) proliferation studies using MTS assay were conducted. BSA-loaded porous structures were obtained on chitosan- and chitosan/gelatin-containing surfaces after freeze-drying, while smooth surfaces were obtained after air-drying. The BSA release rate was directly correlated with increasing gelatin amount in the chitosan/gelatin coatings. MTS analysis was not conclusive because of the absorption properties of polymer coatings. However, absorbed color density in chitosan/gelatin (G:C = 1:1) polymers under freeze-drying conditions was more dominant, indicating better cell proliferation. This method may be used to release growth factors for controlled cell proliferation and differentiation or for the local delivery of antimicrobial drugs to prevent contamination during implementation in hard tissue applications.

Keywords Titanium · Chitosan · Gelatin · Bovine serum albumin · Osteoblast · Hard tissue implants

1 Introduction

Surface properties are the key factors that affect the success of hard tissue implant materials [1, 2]. Various techniques have been used to modify the surface of titanium-based materials, which are often used in hard tissue applications, for better biocompatibility, cell proliferation, osteointegration, and corrosion resistance properties [3–5]. Functional coatings are one of the most commonly preferred techniques. For instance, TiN film coatings are used to increase the corrosion resistance and biocompatibility of titanium-based implant materials [6, 7]. Hydroxyapatite (HA), a natural component of bone, can also be used as a functional coating material to improve the osteointegration properties of hard tissue implant materials [8]. The usage of nanoparticles [9], carbon nanotubes [10], and metal-binding peptides [11] has also been reported.

Natural and synthetic polymers can also be used to modify the surface of titanium and titanium alloys. Drug-loaded poly(lactic-co-glycolic acid) microspheres immobilized on titanium surfaces have been reported to improve cell proliferation [12]. Chitosan-based coatings/microspheres on titanium substrates have been used in the controlled release of both drugs and proteins because of their biocompatibility, biodegradability, and non-toxicity [13]. Gelatin, a natural polymer derived from collagen, has a high degradation rate

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but its debris is non-toxic and positively affects cell proliferation. Chang et al. showed that cell attachment and proliferation were improved by the controlled release of biomolecules from surfaces containing gelatin-based hydrogels [14]. Composite polymers (e.g., chitosan, gelatin, and fibroin at various ratios) may improve cell proliferation and lead to more controlled drug/protein release on hard tissue implant materials. BSA is one of the most widely preferred model compounds for determining the release profile of proteins from these polymers [15].

Porosity is an important parameter that affects the biocompatibility and osteointegration properties of a biomaterial. Cell culture conducted on porous surfaces resulted in better cell proliferation and osteointegration [16]. Various techniques have been used to obtain porous structures on the surface of biomaterials [17]. Deposition of HA coatings onto titanium surfaces is widely used in hard tissue applications because of their biocompatibility and porous structure. Cells and nutrients can easily diffuse through these pores and new tissue generation can start on the surface of the implant material. Various polymers have been used to obtain porous structures on titanium samples, especially using the freeze-drying method [18].

In this study, BSA release from chitosan- and chitosan/gelatin-coated titanium surfaces was studied. For this purpose; (i) BSA-loaded chitosan and chitosan/gelatin coatings were formed under various drying conditions on titanium surfaces, (ii) surface characterization of the surfaces was conducted using Fourier transform-infrared spectroscopy (FTIR) and scanning electron microscopy (SEM), and (iii) BSA release studies in PBS (0.1 M, 37 °C) were conducted, and (iv) a cell viability assay (MTS) was done to determine the effect of porosity and polymer composition on cell proliferation.

2 Materials and Methods

2.1 TiO_2 Formation on Titanium Substrates

Titanium substrates (15 × 15 × 1 mm) were first polished using 1000, 2500, and 4000-grit SiC papers, and then cleaned in acetone and ethanol for 10 min in an ultrasonic bath. Samples were then incubated in 5 M NaOH solution for 24 h. 2 M HCl solution was applied for neutralization for 1 min. The samples were then washed with distilled water. Finally, heat treatment was conducted. The samples were incubated in an incubator for 24 h at 200 °C.

2.2 Salinization of Oxidized Titanium Substrates

Firstly, 10 % (w/v) 3-(triethoxysilyl)-propylamine (3TP) solution was prepared (pH 3). The temperature of the prepared solution was then set to 100 °C on a hot plate. The oxidized titanium substrates were incubated in this solution for 3 min. After the salinization process, the substrate surfaces were rinsed with distilled water and dried at room temperature. The immobilization procedure used in the study is based on covalently binding the $–NH_2$ groups of pure chitosan to the carboxyl groups of the surfaces by using N-(3-dimethylaminopropyl)-N′-ethyl-carbodiimide (EDC) and N-hydroxy-succinimide (NHS). For this purpose, the surfaces were exposed to 8 % glutaraldehyde (GA) solution for 24 h to obtain –COOH groups after the salinization process, as shown in Fig. 1.

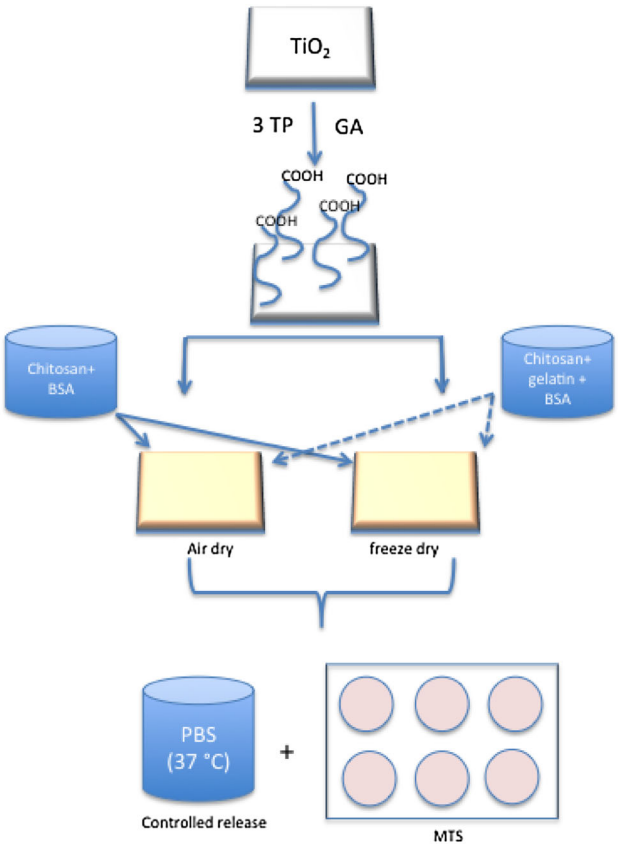

Fig. 1 Proposed surface modification of titanium substrates

2.3 Formation of BSA-Loaded Chitosan and Chitosan/Gelatin Coatings on Titanium Surfaces

BSA-loaded chitosan film formation was optimized in 12-well plates. For this purpose, various chitosan solutions were prepared (1–3 %) by dissolving low-viscosity chitosan polymers (Sigma 50494) in 0.1 M acetic acid. Then EDC/NHS (2:1/mol:mol) for polymer immobilization and bovine serum albumin (BSA) (130 µg/substrate) as a model protein were added to the polymer solution and

mixed. The prepared solution was spread (200 µL/substrate) onto the titanium surface. The samples were then kept at 4 °C for 6 h for immobilization. Finally, the samples were either incubated at room temperature for the air-drying process or first incubated at −20 °C for 20 min and then freeze-dried. 1 % gelatin solutions were prepared at 55 °C using distilled water and mixed with optimized chitosan at various ratios for the preparation of chitosan (C)/gelatin (G) coatings (G:C = 0.5:1, 1:1, 1:0.5).

2.4 Characterization of Titanium Substrates

The surface modification of titanium substrates is summarized in Fig. 1. SEM and FTIR analyses were conducted after surface modification studies to determine the surface morphology and functional groups, respectively.

2.5 BSA Release Studies

The dialysis method was used to determine BSA release rates from the surfaces. Firstly, BSA-loaded surfaces were incubated in 4 ml of PBS (0.1 M, pH 7.4) at 37 °C. 200 µl of a sample were taken from the solution at various time intervals (1–192 h) and replaced with 200 µl of fresh PBS. BSA amounts were determined via ultraviolet (UV) spectrophotometry at 280 nm. Release studies were conducted on both chitosan- and chitosan/gelatin-containing formed under air- and freeze-drying conditions. The calibration curve used to calculate the released BSA amount is shown in Fig. 2.

2.6 MTS Analysis

In order to determine the cell proliferation on different coatings, MTS analysis was conducted. Firstly, the samples were exposed to UV (3 h) for sterilization and then placed into 12-well plates containing the cell culture medium prepared with 50 ml of fetal bovine serum, 1 ml of primocin (InvivoGen, USA), and 500 ml of Dulbecco's modified Eagle medium (DMEM, Nutrient Mixture F-12 HAM)(Sigma, Germany). Human fetal osteoblastic cells (hFOB; American Type Culture Collection (ATCC CRL-11372)) were seeded (1000 cells/sample) on top of the samples and incubated in 5 % CO_2 at 37°C. The medium was refreshed every 3 days and the proliferation of cells on the surfaces was assessed using the MTS cell proliferation assay (96® AQueous Non-Radioactive Cell Proliferation Assay, Promega, USA) for 1–7 days.

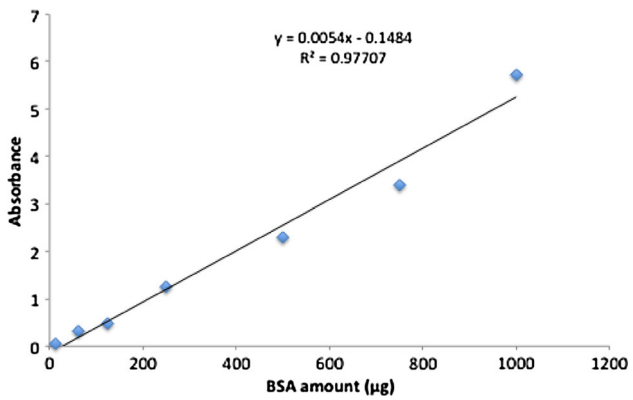

Fig. 2 Calibration curve of BSA

3 Results

3.1 SEM Analysis of Chitosan Sponges Before Coating Process

The chitosan sponges obtained after the air-drying process had a smooth surface without any detectable porosity, as shown in SEM images (data not shown). In contrast, a highly porous structure was obtained for chitosan sponges after the freeze-drying process, as shown in Fig. 3 (cross-sectional view of chitosan sponges).

3.2 SEM Analysis of BSA-Loaded Polymer Coatings on Titanium Substrates

SEM images of chitosan- and chitosan/gelatin-coated titanium surfaces are shown in Fig. 4. No porous structure was obtained after air-drying on either the chitosan- (Fig. 4a) or chitosan/gelatin-containing (Fig. 4b) titanium surface. In contrast, a porous coating formed on both types of surface after freeze-drying (Fig. 4c, d). Since the polymers were immobilized onto the titanium surfaces, cross-sectional views were not obtained. The internal porosity was probably similar to that shown in Fig. 1.

3.3 FTIR Analysis

FTIR spectra of the surfaces after modification are shown in Fig. 5. No functional groups were present on the bare titanium surfaces. After the surface modification process, groups related to BSA (amide II band, at 1518 cm^{-1}) and chitosan (at 1071 cm^{-1}, indicating vibration of CO–C bond, and at 3430 cm^{-1}, indicating vibration of N–H) [19] were obtained.

3.4 BSA Release from Surfaces

Differences were observed in the release behavior of BSA from the surfaces prepared under air-drying conditions. These surfaces were prepared with different gelatin amounts to determine the effect of gelatin on the BSA

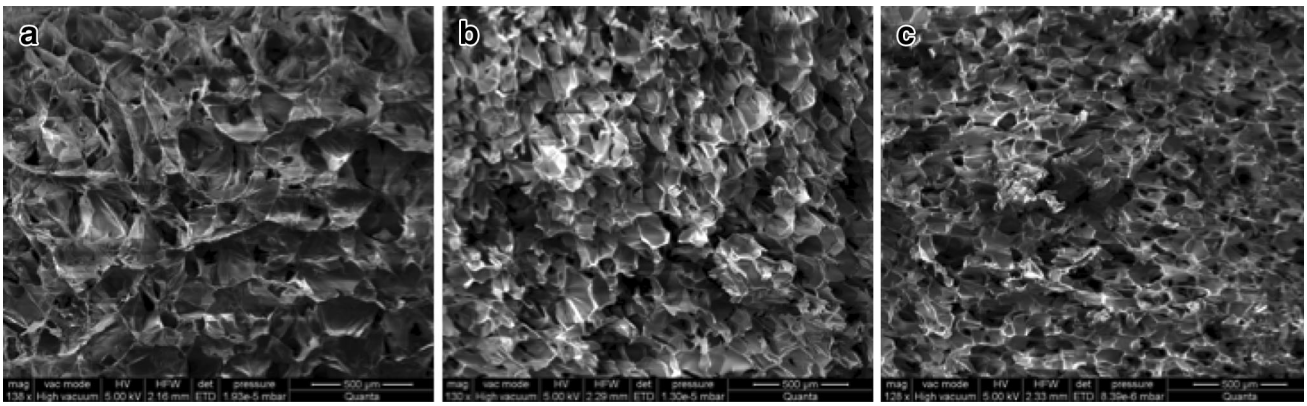

Fig. 3 Cross-sectional SEM images of chitosan sponges prepared using various chitosan concentrations after freeze-drying process in well plate. **a** 1 %, **b** 2 %, **c** 3 %

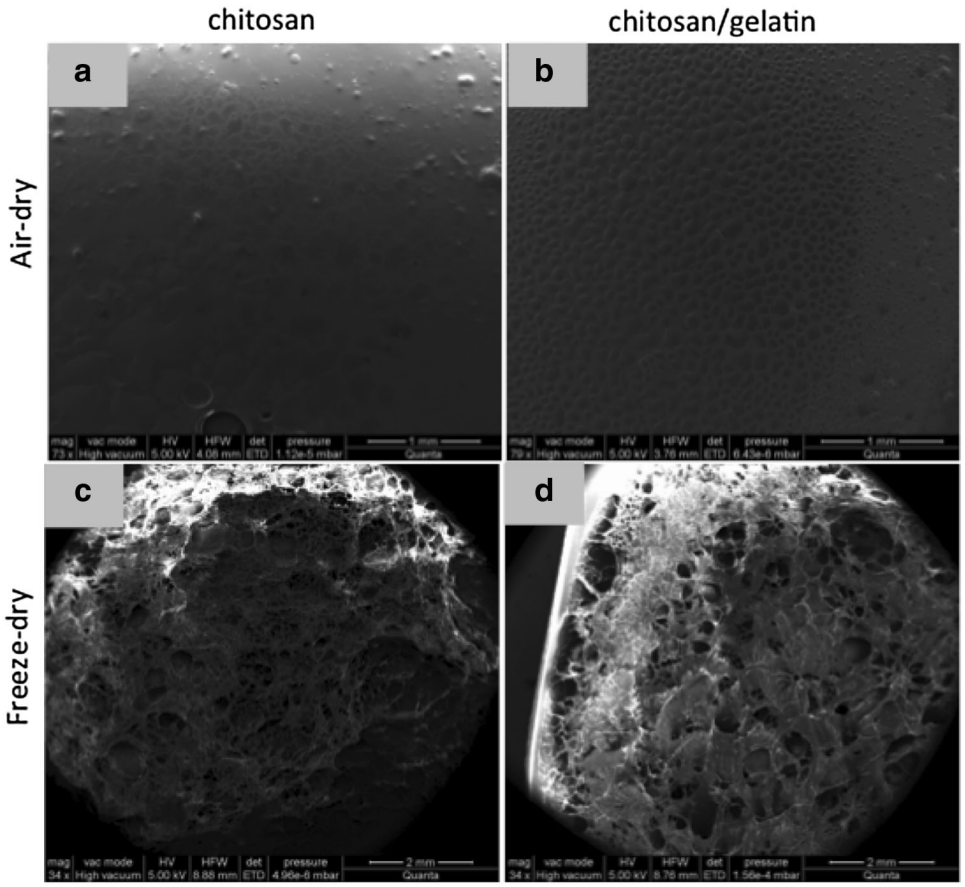

Fig. 4 SEM analysis of polymer-coated titanium surfaces. **a**, **c** Chitosan; **b**, **d** chitosan/gelatin-containing surfaces obtained with air-and freeze-drying, respectively

release rate. The surface with the lowest gelatin amount (G:C/0.5:1) had the lowest BSA release rate (Fig. 6a). An increase in the gelatin amount (G:C/1:1, G:C/1:0.5) resulted in an increase in the BSA release rate. Surfaces with a high gelatin amount had release profiles similar to that for the surfaces that contained only chitosan.

The BSA release profiles for the surfaces prepared under freeze-drying conditions were different from those for the surfaces prepared under air-drying conditions (Fig. 6b). The BSA release rate from the chitosan-containing surfaces was the highest (95 % release in 192 h). The amount of released BSA was also higher compared to that for chitosan coatings prepared by air-drying (70 % release in 192 h). The surface with the lowest gelatin ratio (G:C/0.5:1) had the lowest BSA release rate, as in the air-drying conditions. An increase in the gelatin ratio resulted in an increase in

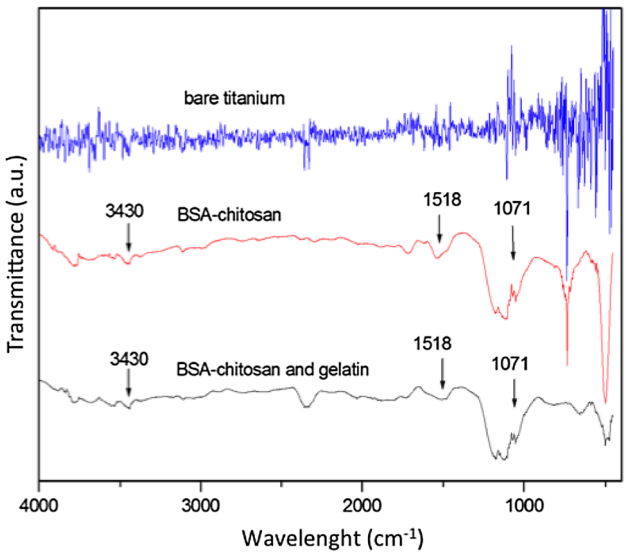

Fig. 5 FTIR spectra of surfaces after modification

the BSA release rate, as in Fig. 6a. The durability of the polymer-coated surfaces was observed for 2 weeks, with no disintegration of the polymer coatings found.

3.5 MTS Analysis

The cell proliferation assay did not give conclusive results because of the absorption of the colored medium by the polymers on the surfaces. The color change (purple) of samples depending on cell activity is shown in Fig. 7 both for the chitosan- and chitosan/gelatin-containing surfaces.

4 Discussion

The SEM analysis clearly showed that the drying method used to form polymeric films on the titanium surfaces directly affects the formation of porous structures, which are known to improve the osteointegration of implanted materials with the surrounding tissue. Moreover, these structures increase roughness, which in turn positively affects cell proliferation [20], and enables cells to migrate toward the surface of implant materials. This migration leads to the formation of new tissue directly on the surface of the implant material [21]. In this study, porous structures on the titanium substrates were obtained both on chitosan- and chitosan/gelatin-coated surfaces. Being a biocompatible polymer, gelatin may further improve the effect of the formed porous structures in terms of cell attachment and migration. Therefore, not only the BSA (or growth factor) release properties of the surface but also the type of polymer used and porosity affect the fate of an implant in the body. Since 2 % chitosan solution resulted in more homogenous porous structures in the cross-sectional SEM analysis (Fig. 3b), it was used in the rest of the study.

FTIR analysis can be used to determine the functional groups formed after modifications on the surface of implant materials [19]. Protein- and chitosan-specific bands in FTIR analysis showed that BSA-loaded chitosan and chitosan/gelatin coatings formed on the surfaces of titanium substrates (Fig. 5).

In vitro release studies showed that BSA release is higher in samples prepared using only chitosan, both after air- and freeze-drying processes. The released BSA amount changed depending on the gelatin amount on the surface. A low amount of gelatin resulted in a low BSA release rate (G:C/0.5:1); increasing the amount increased the release rate. Gelatin is more hydrophilic than chitosan and its degradation rate is higher [22]. Therefore, it is expected that the BSA release rate will be higher from surfaces composed of chitosan/gelatin compared to that of chitosan-containing ones. The reverse trend observed in this study might be related to the crosslinking of carboxyl groups of chitosan and the amino groups of gelatin due to EDC/NHS usage. Thus, gelatin presence probably increased the crosslinking density, slowing down the BSA release from

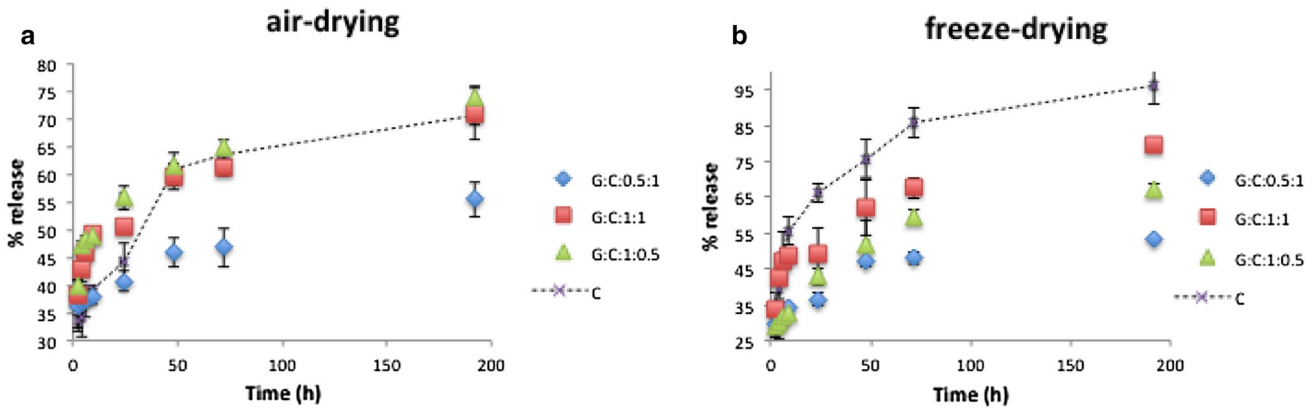

Fig. 6 BSA release from polymer-coated surfaces prepared under **a** air, **b** freeze-drying conditions

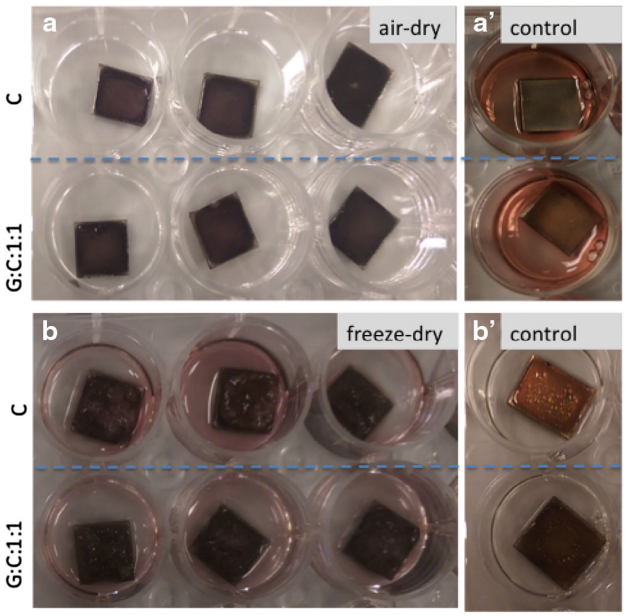

Fig. 7 Absorption of colored medium by polymers during MTS analysis (4 days). **a**, **a'** chitosan; **b**, **b'** chitosan/gelatin coating surfaces obtained with air and freeze drying, respectively

the surface, as shown in Fig. 6. When the gelatin amount in the chitosan/gelatin mixture was increased, however, BSA release increased as expected. BSA release from gelatin coatings was also attempted, but the structural integrity of this layer was rapidly disturbed probably because of the high dissolution rate of gelatin in water and its limited mechanical stability (data not shown) [22]. Another finding was that the amount of released BSA changed depending on the drying method used. The released BSA amount was 70–95 % (Fig. 6b) when freeze-drying was used. This is probably due to the increased porosity of the surfaces, leading to more BSA release. Release studies showed that both chitosan and chitosan/gelatin coatings formed on titanium samples after air- and freeze-drying, which can be used to adjust the protein release rate according to the desired goals. Although BSA was used in this study as a model protein in the controlled release studies [15], this method may be applied for the release of growth factors to enhance cell proliferation [23] or for antibiotic release to prevent contamination during implementation [24].

The MTS assay is a colorimetric detection method used to determine cell viability. As a consequence of the metabolic activity of cells, tetrazolium in the assay medium causes a change in the color of the cell culture medium [25]. Purple color formation was observed in this study, but a quantitative result of the cell number could not be obtained because of the absorption properties of polymer coatings. The colored medium was absorbed by the biopolymers (Fig. 7), resulting in fluctuations in color intensity. However, the color intensity observed by the naked eye showed that cell attachment was successfully achieved on all surfaces and that cell proliferation was best on chitosan/gelatin-containing surfaces prepared with the freeze-drying process (Fig. 7).

5 Conclusion

In this study, BSA release from chitosan- and chitosan/gelatin-coated titanium surfaces was studied. Surfaces were prepared under air- and freeze-drying. Smooth surfaces were obtained with air-drying, whereas porous structures formed when surfaces were subjected to freeze-drying. The drying method not only affected porosity, but also the amount of released BSA. Low amounts of gelatin in chitosan/gelatin coatings resulted in a low rate of BSA release; increasing the gelatin amount in coatings resulted in an increase in the BSA release rate. Cell proliferation assay studies (MTS) did not give quantitative results because of the absorption properties of polymer coatings (i.e., the polymers absorbed the colored medium, affecting the color intensity measurement results). However, the absorbed color density was highest on chitosan/gelatin-containing surfaces prepared under freeze-drying conditions, indicating better cell proliferation. This method may also be used to release growth factors for improved cell proliferation, or in drug release studies to prevent contamination during implementation for hard tissue applications.

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