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Evaluation of the effects of Ag ion concentration on osteoblast activity

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Abstract. We reported the apatite-forming ability of 30CaO·70SiO_2 scaffolds with 0~100 ppm Ag ions by soaking in simulated body fluid (SBF). This study was to evaluate the effects of the concentrations of Ag ions in the 30CaO·70SiO_2 gels on *in-vitro* biocompatibility of osteoblasts (MC3T3). After seeding cells on the surface of Ag-30CaO·70SiO_2 gels scaffold, cellular behaviors were evaluated by an assay of cell counting kit-8. Cytotoxicity of the scaffold samples was evaluated by employing the extract solutions of the scaffold samples by the assays of neutral red, MTT and BrdU. In addition, live & dead assay was performed by using a gel covering method, which the scaffolds have been directly contacted with the incubated cells on the well plate. According to the results of CCK-8 assay, the optical density value of the absorbance of the resulting solution decreased as the concentration of Ag ions in the scaffolds increased. Moreover, their cell viability was measured to be less than 50% at the Ag concentrations of 50 ppm or more, and dead cells were observed in the experiment results of both the cytotoxicity and gel covering tests. From these experimental results, we concluded that the Ag-30CaO·70SiO_2 scaffolds with less than 50 ppm Ag ion concentration were considered as biocompatible.

Introduction

Ceramic biomaterials have been used to recover the lost functions of the damaged bones inside the body by directly fusing with the bones. 30CaO·70SiO_2 gel has been known as a bioactive material that has an ability to form apatite on the surface of bones inside the body fluid [1]. We reported in previous that spherical particles of 30CaO·70SiO_2 gel can be made by using a sol-gel method [2], which has two merits such as a capability to synthesize constant and even high-purity material, and a capability to easily manipulate it with desired shapes [3]. However, applications of biomaterials could require use of excess amount of antibiotics during transplantation to prevent infection or microbism. Ag ions have been recognized as its unique biological properties of antibiosis. There is a need to manufacture a new biomaterial with Ag ion to directly prevent generation of any infection. In this study, after synthesizing 30CaO·70SiO_2 gel with 10-100 ppm Ag ion using our previous sol-gel method, it was deposited into the simulated body fluid (SBF) to observe formation of apatite [4]. Furthermore, we studied its biocompatibility using the Ag-30CaO·70SiO_2 gel as a scaffold.

Experimental

After preparing for 10-100 ppm of Ag standard solution as suggested by a vendor (KANTO Chemical CO., INC., Japan), polyethylene glycol (PEG, Aldrich) with MW of 10,000 and $Ca(NO_3)_3 \cdot 4H_2O$ were dissolved into the standard solution [2]. Nitric acid (62 wt %; Wako, Japan) was added as a catalyst to the solution. During vigorously stirring of the obtained solution, tetraethoxysilane (TEOS) was added and stirred for 20 min. The mixture solution was gelatinized inside the 40℃ oven for 24 hrs. Dried gel was heated in 800℃ and maintained for 2 hrs. The obtained gel was deposited in a container with SBF solution, with a dimension of 10 mm x 10 mm x 1 mm to induce apatite formation. The surface of the obtained gel was analyzed with the methods of x-ray diffraction (XRD) and a scanning electron microscope (SEM).

Biocompatibility of the gel was performed using *in vitro* cell-culture after sterilizing Ag-$30CaO \cdot 70SiO_2$ gel with ethylene oxide gas. Furthermore, CCK assay was done at the days of 1, 3, 7 after inoculating MC3T3 (100,000 cells/surface) on the surface of the scaffold, and live & dead assay was done on day 7 and 14 using fluorescence microscope (Leica DMLB, Germany). Moreover, the optical densities of the absorbance with reference wavelengths of 550 nm and 690 nm was measured using micro plate reader after seeding the osteoblast on the plate with the effluent from the scaffold for 72 hrs. Cytotoxicity of the scaffolds was tested by measuring the absorbance at the wavelength of 550 nm (R: 690 nm) 2 hrs after addition of 0.33% neutral red solution to cells. For MTT assay, absorbance of extracts at the wavelength of 570 nm was measured 4 hrs after loading the thiazoly blue tetrazolium 0.002 g/ml dissolved in 1 mL PBS buffer. BrdU assay was also performed by measuring the optical density of the extracts at the wavelength of 450 nm (R:690) 2 hrs after loading BrdU solution (1:100 solution) in to the cell culture medium.

Results and discussion

As shown in Figure 1, behaviors of apatite formation on the surface of the $30CaO\text{-}70SiO_2$ gel with no Ag ions added were observed before and after its deposition in SBF. The apatite surrounded the porous surface of the gel on the first day of its deposition in SBF, and addition of different concentrations of Ag ions did not affect the formation of the apatite, showing similar morphologies of Figure 1-b. The concentrations of Ag ions added were 10, 20, 50, 75 and 100ppm (Figure 2-a and b). Based on the results of XRD analyses as shown in Figure 2, hydroxyapatite crystalline was observed at 2 theta 25.8° and 31.7° peak on every gel after 1 day of deposition in SBF.

Figure 3 shows the result of biocompatibility of the gel scaffolds. The absorbance of formazan, which was formed in the process of returning the material that was generated from the mitochondrial respiration on the MC3T3, was measured by performing the CCK-8 assay to the cells on the surfaces of the scaffolds. For comparison of cell responses to the gels with different amount of Ag ions, collagen-coated gel was employed as a positive control. By coating collagens, the gel induced significant amount of cell adhesion and proliferation (Figure 3-a). As the contents of Ag ions increased, the degree of cell adhesion was reduced. Furthermore as cell culture lasted to 14 days, degree of cell adhesion increased, however, cell proliferation did not increase for the samples with higher amount of Ag ions. Figure 3(b) showed that the rate of cell proliferation decreased as the Ag content increased, by normalizing the optical density value of the adhered cells at day 1. The rates of cell proliferations were higher for the samples with lower amount of Ag ions than those with higher amount of Ag ions. In specific, it appeared that while the gel scaffolds induced lower cell adhesion and proliferation when 75 ppm or above Ag ions were added, those did higher cell adhesion when 50 ppm or below of Ag ions were added.

Cell survival and proliferation were observed by fluorescence microscope at day 7 and 14 (Figure 4). The positive control samples which did not have any Ag ions and were coated with collagen showed excellent cell adhesion and survival (Figure 4-a, e). Nearly all the cells seemed to be survival on the gels with low amount of Ag ions added as shown in green(Figure 4-b, f), but very small number of dead cells was observed in red on the gels with Ag ions added (Figure 4-c, d, g, h). Numerous viable cells were found on the gel with Ag ions 50 ppm or below, but cell proliferation

could not be achieved on the gel with Ag 75 ppm or above, possibly due to the effects of Ag ions on cell adhesion and proliferation, indicating the properties of cell non-adhesiveness of Ag ions.

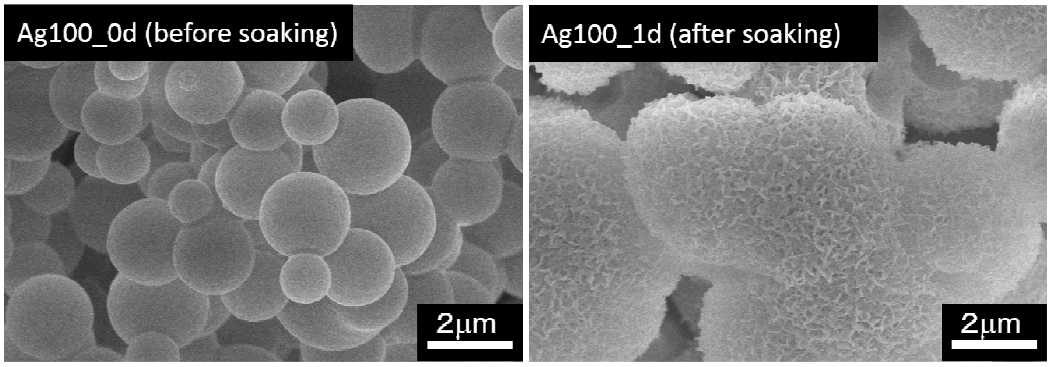

Figure 1 Apatite-forming ability of $30CaO{\cdot}70SiO_2$ gel with addition of 100ppm Ag ion after deposition in SBF observed using a SEM.

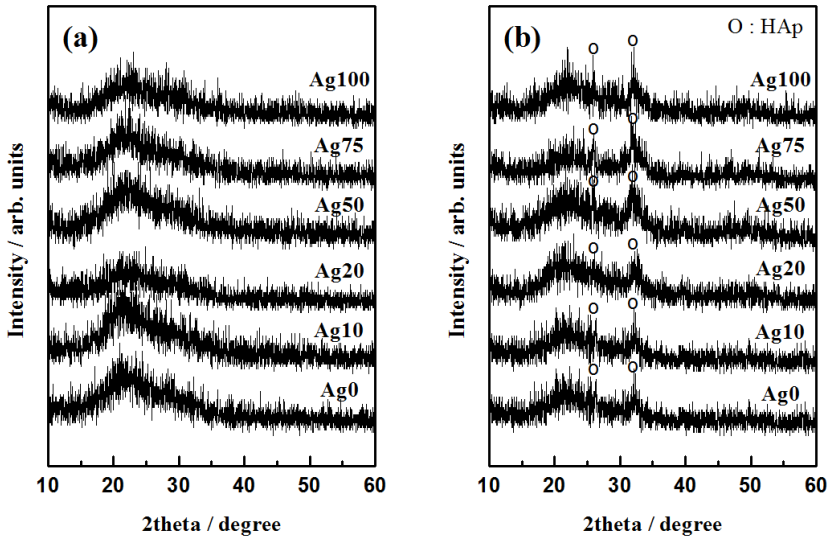

Figure 2 XRD patterns of Ag ion solution inside the $30CaO\text{-}70SiO_2$ gel (a) before the deposition and (b) 1 day after the deposition.

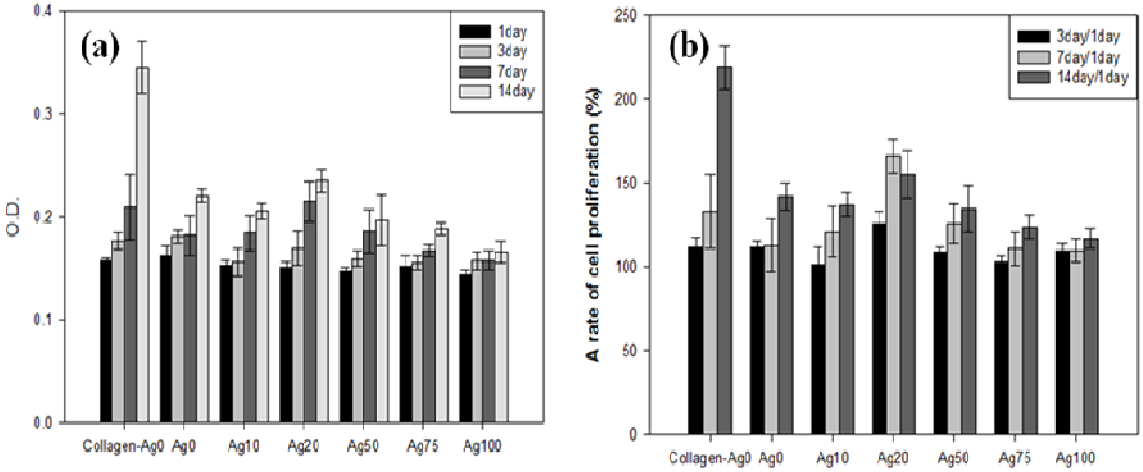

Figure 3 Results of CCK-8 assays of (a) cell-adhesiveness and (b) proliferation of MC3T3 cell on the surfaces of Ag-$30CaO{\cdot}70SiO_2$ gel scaffolds.

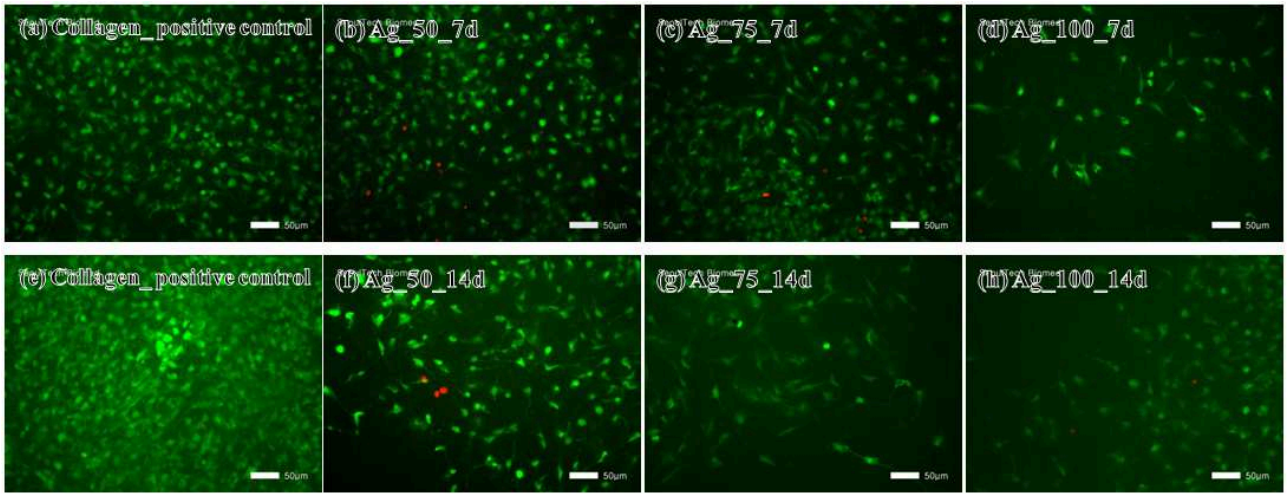

Figure 4 Result of MC3T3 cell on the surface of gel scaffold measured at day 7 and 14 by the method of Live & Dead assay (x200).

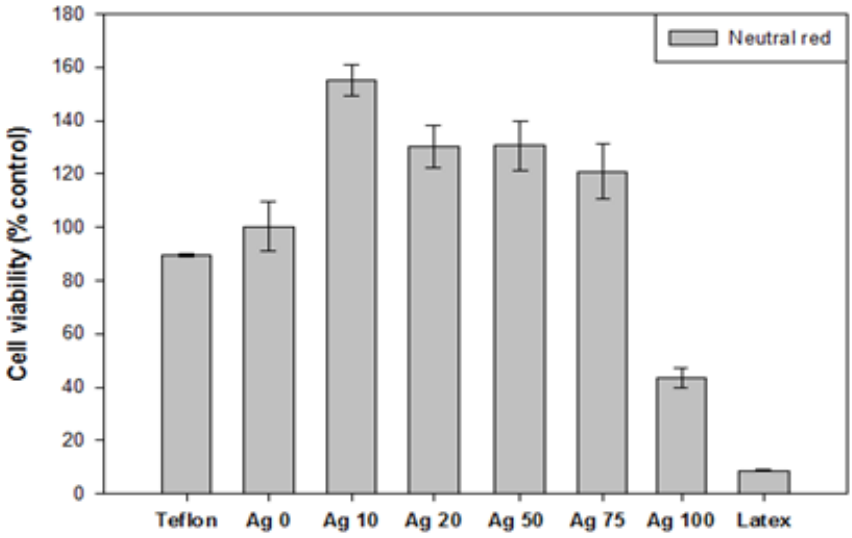

Figure 5 Result of Neutral red assay using Microplate reader.

Figure 5 showed the results of cell survival on the gel surfaces with different amount of Ag ions by employing Teflon and Latex as positive and negative controls with Neutral red assay. While Teflon has been considered as a polymer not releasing any low molecules, Latex as a polymer releasing some toxic molecules. The cell viability of Teflon was considered as 100% and those of all other gel samples were compared with. The gels containing Ag ions upto 75 ppm showed higher degrees of cell viability but that with 100 ppm showed significant less amount of cell viability but higher than that of Latex.

Based on the abover evaluation results by CCK-8, BrdU and SEM, the gel containing less than 50 ppm Ag ions demonstrated higher cell adhesion, proliferation and viability. Therefore, introduction of Ag ions should be below 50ppm for induction of cell adhesion. But when we aims to limit cell adhesion, i.e. repulsion of cells on the gel surface, introduction of Ag ions to gel should be higher than 75 ppm, even though we employed MC3T3 bone cells for the tests of cell adhesion behaviors.

Summary

We farbricated Ag-30CaO·70SiO_2 gels with different amount of Ag ions, and the apatite was formed during their deposition in SBF regardless of concentration of Ag ion. Based on the results of cellular responses on the gels with Ag ions, the gels with more than 50 ppm Ag ions seemed to induced MC3T3 bone cells repellence but still have high cell interaction with the surfaces. Likewise, appropriate concentration of Ag ion additive as an antibiotic material would be 50 ppm or higher, and its biocompatibility seemed to be kept by using the gel with incorporation of Ag ions at the concentrations of 50 ppm or below.

Acknowledgements

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