

Research Article

Effects of Si(IV) Released from Chitosan-Silicate Hybrids on Proliferation and Differentiation of MG63 Osteoblast Cells

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Abstract Chitosan- γ -glycidoxypolytrimethoxysilane (GPTMS)-tetraethoxysilane (TEOS) hybrid membranes were prepared by a sol-gel method. Effects of Si(IV) released from them on proliferation and differentiation were examined in terms of cell metabolic activity and alkaline phosphatase (ALP) activity of MG63 osteoblastic cells. The amount of Si(IV) released from the hybrid membranes increased with the TEOS content. Released Si(IV) inhibited cell proliferation, while it promoted cell differentiation. Thus, osteocompatibility of the chitosan hybrid membranes can be controlled due to the amount of Si(IV) released from them as the hybrids in the system chitosan-GPTMS-TEOS are applied for cell culture.

Keywords chitosan; organic-inorganic hybrid; Si(IV); cell proliferation; osteodifferentiation

1 Introduction

Organic-inorganic hybrids are controllable not only in rate of bioresorption or biodegradation but also in cell response, which ensures them to be a good candidate for tissue engineering scaffolds. Chitosan, a mucopolysaccharide having structural characteristics similar to glycosamines, is obtained from alkali-deactivation of chitin derived from exoskeleton of crustaceans [2]. Chitosan is biodegradable, biocompatible, non-antigenic and non-toxic and then has been studied for use in a number of biomedical applications. Shirosaki et al. [6,7,8] found that hybrids of chitosan and γ -glycidoxypolytrimethoxysilane (GPTMS, $\text{CH}_2\text{OCHCH}_2\text{OCH}_2\text{CH}_2\text{Si}(\text{OCH}_3)_3$) were cytocompatible in terms of human osteosarcoma cell MG63 and human bone marrow osteoblast (HOB). Addition of minimal content of GPTMS improved adhesion and proliferation of MG63 cells on the chitosan-GPTMS hybrids compared to those on the chitosan membrane. HOB cells proliferated

on chitosan-GPTMS hybrids formed a fibrillar extracellular matrix with numerous calcium phosphate globular structures in the absence of dexamethasone. They also confirmed that their biodegradability was controllable due to the GPTMS content [10]. Recently much interest is focused on cell responses to Si(IV) released from materials after Xynos et al. [11] or Hench and Polak [4] reported that Si(IV) affected cells proliferation or DNA representation. Tsuru et al. [9] prepared hybrids in the system gelatin-GPTMS-tetraethoxysilane (TEOS) with varied GPTMS contents and pointed out that Si(IV) and Ca(II) released from the hybrids affected osteoblast differentiation. Those results indicate that Si(IV) from the chitosan-GPTMS hybrids enhances osteocompatibility. In this study, the Si(IV) was enriched in the chitosan-GPTMS hybrids by adding TEOS to the precursor solutions, and that the effects of the Si(IV) release were observed on MG63 cell proliferation and differentiation.

2 Materials and methods

2.1 Preparation and characterization of hybrids

An appropriate amount of chitosan (Aldrich, high molecular weight) was dissolved into 0.25 M acetic acid aqueous solution. At first, GPTMS was added into chitosan solution and the mixture was stirred for 1 h at room temperature. Then, TEOS was also added into chitosan-GPTMS mixture and they were stirred again for 1 h at room temperature. Both GPTMS and TEOS were pre-hydrolyzed with 0.25 M acetic acid aqueous solution for 1 h at room temperature before being added into chitosan solution. The resultant sols were poured into each well of cell culture plates (Falcon, Multi-well TC plates). The plates with sols were stood overnight at room temperature. The resultant sols were dried at 60 °C for 2 days to yield the hybrid membranes. The obtained

hybrid membranes were soaked in 0.25 M sodium carboxylate solution and washed with distilled water to neutralize the remaining acetic acid in them. The FT-IR spectra of the hybrid membranes were measured by the KBr method on an FT-IR300 spectrometer (JASCO, Japan). The data were recorded by the accumulation of 100 scans with a resolution of 4 cm^{-1} . Table 1 shows the sample code for each starting composition.

Sample code	Chitosan	GPTMS	TEOS
Ch	1.0	0	0
ChG05	1.0	0.5	0
ChT05	1.0	0	0.5
ChG05T05	1.0	0.5	0.5
ChG05T10	1.0	0.5	1.0

Table 1: Sample code and starting composition of the hybrid membranes (molar ratio).

2.2 In vitro cytocompatibility and Si(IV) release behavior

The hybrid membranes were sterilized by soaking into 70% ethanol and washed with PBS. Then, they were soaked in MEM α overnight under 5% CO₂ at 37 °C, 95% humidity. MEM α contained 10% fetal bovine serum (FBS), 1% penicillin and streptomycin solution (GIBCO BRL 15140-122), $2.5\ \mu\text{g mL}^{-1}$ fungizone and $50\ \mu\text{g mL}^{-1}$ ascorbic acid. Human osteoblastic cells MG63 were employed for direct biocompatibility evaluation of the hybrid membranes. MG63 cells were cultured ($10^4\ \text{cells cm}^{-2}$) up to 7 days in control conditions (absence of materials, Thermanox (Nalge Nunc international K.K. Tokyo, Japan)) and on the surface of the hybrid membranes. AlamarBlue assay (TREK Diagnostic Systems, OH, USA) was used for the evaluation of cellular viability or metabolic activity. At each culture day, a mixture of AlamarBlue (in an amount equal to 10% of the total culture volume) was aseptically added to the samples. Samples were incubated with AlamarBlue for additional 3 h. AlamarBlue is chemically reduced by the metabolic activity of growing cells, which causes the fluorometric-colorimetric REDOX indicator to change from an oxidized, nonfluorescent blue form to a reduced, fluorescent red form. The fluorescence intensity was measured by using a Multi-Microplate Reader (MTP-800Lab, CORONA ELECTRIC Co., Ibaragi, Japan) with excitation at 550 nm and emission at 615 nm according to the manufacturer's direction. The amount of Si(IV) released into MEM α was measured with inductively coupled plasma emission spectroscopy (ICP7500, Shimadzu, Japan). ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton in water) and assayed by the hydrolysis of *p*-nitrophenyl phosphate in an alkaline buffer solution, pH10, and colorimetric determination of the product (*p*-nitrophenol) at $\lambda = 405\ \text{nm}$.

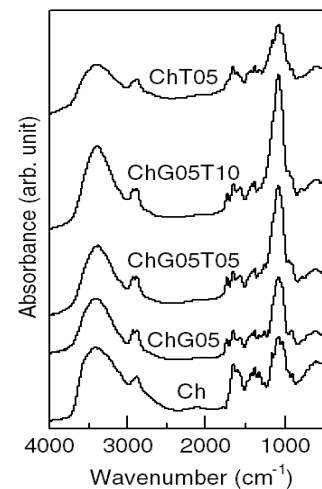


Figure 1: FT-IR absorption spectra of the chitosan hybrid membranes.

3 Results and discussion

As described previously, GPTMS crosslinked chitosan matrix and formed Si-O-Si network in the hybrid matrix. Figure 1 shows the FT-IR absorption spectra of the chitosan hybrid membranes. The spectra of Ch and ChG05 showed the patterns similar to the previous data. The bands denoted as amide I and II, characteristic of the chitosan structure, were detected around 1650 cm^{-1} and 1565 cm^{-1} in the spectra of all membranes. The peaks assigned as methoxy groups (2840 cm^{-1}) derived from GPTMS disappeared in the spectra of the hybrid membranes. Moreover, the hybrid membranes showed $\nu(\text{Si-O})$ bands in $1110\text{--}1000\text{ cm}^{-1}$ region. These bands in ChGT groups became much sharp as compared with Ch and ChG groups. This indicated that TEOS could be hydrolyzed and recondensed to form Si-O-Si network in the hybrid structure in the same way as GPTMS.

Table 2 shows the amount of Si(IV) released from the hybrid membranes into MEM α up to 7 days. As compared with ChG05 and ChT05, the amount of Si(IV) released from ChT05 was higher than that of ChG05. It indicates that chitosan interacts with GPTMS stronger than TEOS. TEOS is hydrolyzed and forms the silanol groups. According to Rashidova et al. [5], the silanol groups with negative charge and the amino groups with positive charge favor the crosslinking reaction. However, this interaction is weaker than GPTMS with both epoxy and silanol groups as the active sites as the crosslinking with chitosan matrix. Therefore, the Si(IV) derived from TEOS in chitosan matrix can be released easily. In the case of ChG05 with TEOS (ChG05T05 and ChG05T10), the amount of Si(IV) released from the hybrid membranes increased as the starting composition of TEOS. This result suggests that the amount of Si(IV) released from the

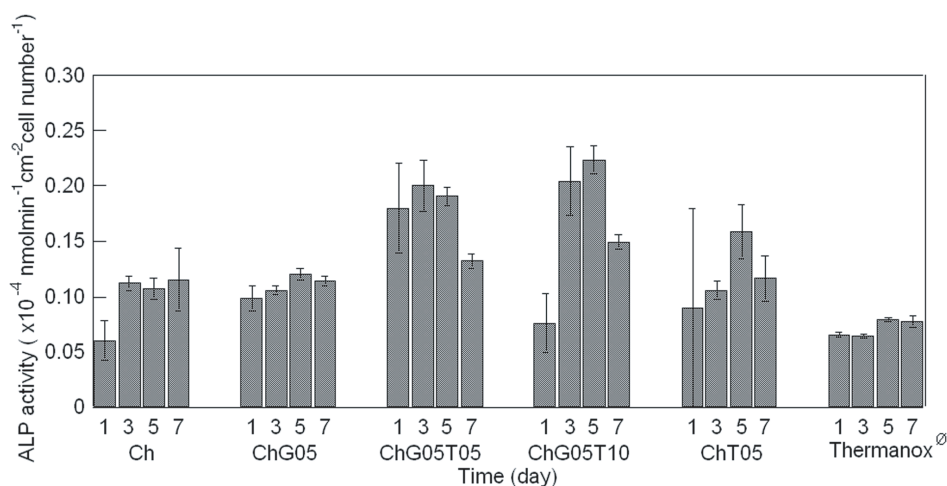


Figure 2: ALP activity of MG63 cells cultured on the hybrid membranes.

hybrid membranes is controllable by the addition of TEOS into chitosan-GPTMS hybrid membranes. MG63 cells proliferated on all membranes. In previous study [8], the cell attachment and proliferation were greatly improved on ChG05 as compared to Ch. The cells cultured on ChT05 hardly grew within 7 days as observed on Ch. For ChG05T05 and ChG05T10, cells grew better than Ch but less than ChG05. This means that Si(IV) released from the hybrid membranes with TEOS inhibited their proliferation. The content of TEOS in ChG hybrid membranes did not affect the cell proliferation. Figure 2 shows ALP activity of MG63 cells cultured on the hybrid membranes. The ALP activity of MG63 cells cultured on Ch, ChG05 and Thermanox was low up to 7 days. On ChT05, the ALP activity increased until 5 days and was a little bit higher than ChG05. While on ChG05T05 and ChG05T10, the ALP activity was significantly higher than ChG05. The release of Si(IV) ions might directly affect cell differentiation. Bielby et al. reported that the amount from 2.1 mM to 5.0 mM Si(IV) by 58S bioactive sol-gel glass in medium promoted the osteodifferentiation [1]. Moreover, Gough et al. found that more than 8.2 mM Si(IV) released from Bioglass in medium caused cell apoptosis [3]. In the present study, when Si(IV) was less than 2.0 mM in 7 days culture, ChG05 caused no effects on the cell differentiation. This means that the Si(IV) released from ChG05 was insufficient to induce cell differentiation. In contrast, cell proliferation on ChT05 was inhibited, and differentiation slightly was promoted although the amount of Si(IV) was more than 2 mM. For ChG05T05 and ChG05T10, whereas the amount of Si(IV) released from them was greater than 8 mM after 7 days, no apoptosis was observed and thus cell differentiation was improved. It is expected that Si species released from chitosan-GPTMS-TEOS hybrid membranes have good potential for the osteodifferentiation.

Sample code	Soaking period (day)			
	1	3	5	7
ChG05	0.47	1.21	1.68	2.07
ChT05	2.62	4.71	5.00	5.04
ChG05T05	1.83	4.32	6.31	8.16
ChG05T10	2.15	5.06	7.96	10.23

Table 2: The amount of Si(IV) released from the hybrid membranes into MEM α (mM).

4 Conclusions

Addition of TEOS improved osteocompatibility of the chitosan-GPTMS hybrid membranes since it provided them a Si(IV) releasing ability: a greater amount of Si(IV) was released from the chitosan-GPTMS-TEOS hybrids than from the chitosan-GPTMS ones. Hence, MG63 cells were much proliferated on chitosan-GPTMS than on chitosan-GPTMS-TEOS. Inverse effects were observed on the ALP activity. The results suggested that not only the amount of Si(IV) but also their species would affect the cell behavior.

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References

- [1] R. C. Bielby, R. S. Pryce, L. L. Hench, and J. M. Polak, *Enhanced derivation of osteogenic cells from murine embryonic stem cells after treatment with ionic dissolution products of 58S bioactive solgel glass*, *Tissue Eng*, 11 (2005), pp. 479–488.
- [2] T. Chandy and P. C. Sharma, *Chitosan—as a biomaterial*, *Biomater Art Cells Art Org*, 18 (1990), pp. 1–24.

- [3] J. E. Gough, J. R. Jonesb, and L. L. Henchb, *Nodule formation and mineralisation of human primary osteoblasts cultured on a porous bioactive glass scaffold*, *Biomaterials*, 25 (2004), pp. 2039–2046.
- [4] L. L. Hench and J. M. Polak, *Third-generation biomedical materials*, *Science*, 295 (2002), pp. 1014–1017.
- [5] S. S. Rashidovaa, D. S. Shakarovaa, O. N. Ruzimuradova, D. T. Satubaldievaa, S. V. Zalyalievaa, O. A. Shpigunb, et al., *Bionanocompositional chitosan-silica sorbent for liquid chromatography*, *J Chromat B*, 800 (2004), pp. 49–53.
- [6] Y. Shirosaki, T. Okayama, K. Tsuru, S. Hayakawa, and A. Osaka, *Synthesis and cytocompatibility of porous chitosan-silicate hybrids for tissue engineering scaffold application*, *Chem Eng J*, 137 (2008), pp. 122–128.
- [7] Y. Shirosaki, K. Tsuru, S. Hayakaw, A. Osaka, L. M. A., et al., *In vitro cytocompatibility of MG63 cells on chitosan-organosiloxane hybrid membranes*, *Biomaterials*, 26 (2005), pp. 485–493.
- [8] Y. Shirosaki, K. Tsuru, S. Hayakawa, A. Osaka, M. A. Lopes, J. D. Santos, et al., *Physical, chemical and in vitro biological profile of chitosan hybrid membrane as a function of organosiloxane concentration*, *Acta Biomat*, 5 (2009), pp. 346–355.
- [9] K. Tsuru, Z. Robertson, B. Annaz, I. R. Gibson, S. M. Best, Y. Shirosaki, et al., *Sol-gel synthesis and in vitro cell compatibility analysis of silicate-containing biodegradable hybrid gels*, *Key Eng Mater*, 361-363 (2008), pp. 447–450.
- [10] K. Tsuru, Y. Shirosaki, S. Hayakawa, A. Osaka, M. A. Lopes, J. D. Santos, et al., *In vitro biodegradability of chitosan-organosiloxane hybrid membrane*, *Key Eng Mater*, 284-286 (2005), pp. 823–826.
- [11] I. D. Xynos, A. J. Edgar, L. D. K. Buttery, L. L. Hench, and J. M. Polak, *Gene-expression profiling of human osteoblasts following treatment with the ionic products of bioglass® 45S5 dissolution*, *J Biomed Mater Res*, 55 (2001), pp. 151–157.