THE EFFECTS OF SBF CONDITIONS ON ENCAPSULATION OF AGAROSE GEL WITH HYDROXYAPATITE MICROCAPSULES

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Abstract: By raising the temperature or the pH of an acellular simulated body fluid (SBF), nanoparticles of amorphous calcium phosphate are precipitated. The nanoparticles are highly active to induce hydroxyapatite formation in body fluid or SBF. In this study, the authors fabricated hydroxyapatite microcapsules encapsulating an agarose gel by using the nanoparticles and examined the effects of the pH and the concentrations of SBF on the microcapsules formation process. Increase in ion concentrations or raising pH of SBF caused the acceleration of the microcapsules formation.

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1. INTRODUCTION

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), the major component of human bones and teeth, is the important bioceramic for medical engineering because of its high bioaffinity and non-toxicity to living bones¹⁻⁴ and cells^{5,6}. In addition, it also performs biopolymer adsorption ability such as a protein adsorption⁷.

The simulated body fluid (SBF) has inorganic ion concentrations nearly equal to those of human blood plasma⁸⁻¹⁰. This fluid is able to be useful to reproduce the reaction of bone-like hydroxyapatite formation in living body.

By raising the temperature or the pH of SBF, nanoparticles of amorphous calcium phosphate are precipitated. Recently, we reported that the nanoparticles actively induce hydroxyapatite formation in SBF or in body fluid, and we named the particle apatite nucleus^{11,12}.

Previous experiments by our group have shown that several hydroxyapatite microcapsules were fabricated by using apatite nucleus^{13,14}. The microcapsules encapsulating Ag microspheres possessed a sustained-release of Ag ion¹⁵. The sustained-release properties were also demonstrated in the case of encapsulating silica gel microspheres inducing insulin¹⁶ and agarose gels inducing vitamin B₁₂¹⁷. The hydroxyapatite microcapsules encapsulating maghemite microparticles possessed a

protein adsorption property and was able to be collected by magnetism of maghemite¹⁸. The microcapsules encapsulating plasmid DNA were successful in a gene transfer^{19,20}.

Thus using apatite nucleus made it possible to fabricate various hydroxyapatite microcapsules in SBF. It is considered that the formation process of hydroxyapatite microcapsules is based on the following procedure. Firstly, apatite nuclei are attached to or included in microparticles. Secondly, the microparticles are soaked in SBF. The apatite nuclei on the surface of the microparticles encourage the formation of hydroxyapatite. Hydroxyapatite is induced on their surface in SBF, and the microcapsules are formed.

Although the fabrication of hydroxyapatite microcapsules was studied, the effects of SBF conditions on the formation of the microcapsules have not been examined. Generally, hydroxyapatite formation in an aqueous solution is affected by the pH values and the ion concentrations because these factors are closely related to the degree of supersaturation of the solution against hydroxyapatite. In this study, the pH or ion concentrations of SBF were changed and their effects on the formation of hydroxyapatite microcapsules encapsulating agarose gels was investigated.

2. MATERIALS AND METHODS

Three types of SBF (NM-SBF, HP-SBF, HC-SBF) with the pH value and the ion composition shown in TABLE 1 were prepared in order to examine the effects of the pH and the ion concentration of SBFs. TABLE 2 shows the amount of volume of the reagents dissolved in ultrapure water for preparation of each types of SBF. The reagent grade NaCl, NaHCO₃, KCl, K₂HPO₄·3H₂O, MgCl₂·6H₂O, 1M-HCl, CaCl₂, Na₂SO₄ whose amount or volume is shown in TABLE 2 were dissolved in ultrapure water in order and transferred into a 1 L measuring flask. The pH value of SBFs shown in TABLE 1 was controlled by dissolving tris(hydroxymethyl)aminomethane.

TABLE 1 Ion concentrations of human blood plasma and SBFs

	Ion concentrations / mM			
	Human blood plasma	NM-SBF (normal)	HP-SBF (high pH)	HC-SBF (high conc.)
Na ⁺	142.0	142.0	142.0	343.9
K ⁺	5.0	5.0	5.0	15.0
Mg ²⁺	2.5	1.5	1.5	3.6
Ca ²⁺	1.5	2.5	2.5	7.5
Cl ⁻	103.0	147.8	147.8	359.6
HCO ₃ ⁻	27.0	4.2	4.2	12.6
HPO ₄ ²⁻	1.0	1.0	1.0	3.0
SO ₄ ²⁻	0.5	0.5	0.5	1.5
pH (36.5 °C)	7.20-7.40	7.40	7.60	7.00

TABLE 2 Amounts of reagents for preparing 1 L of SBFs

	Amount / volume of reagents		
	NM-SBF (normal)	HP-SBF (high pH)	HC-SBF (high conc.)
NaCl	7.996 g	7.996 g	19.19 g
NaHCO ₃	0.350 g	0.350 g	1.050 g
KCl	0.224 g	0.224 g	0.672 g
K ₂ HPO ₄ ·3H ₂ O	0.228 g	0.228 g	0.684 g
MgCl ₂ ·6H ₂ O	0.305 g	0.305 g	0.732 g
1M-HCl	35 mL	35 mL	105 mL
CaCl ₂	0.278 g	0.278 g	0.834 g
Na ₂ SO ₄	0.071 g	0.071 g	0.213 g

Apatite nucleus was prepared by raising the pH of NM-SBF to 8.60 dissolving tris(hydroxymethyl)aminomethane at 25.0 °C. The

precipitated apatite nuclei were collected by filtration using a nitrocellulose membrane filter (MF-Millipore, Millipore Corporation, USA) of 50 nm pore size.

Sixty mg of an agarose (Agarose H, Nippon Gene Co., Ltd., Tokyo, Japan) and 2 mg of apatite nuclei were added in 10 mL of ultrapure water. The agarose was dissolved in the water by boiling in the microwave. Then apatite nuclei were dispersed in thus obtained agarose sol by ultrasonic vibration for 10 minutes at 38.0 °C. Each SBF (NM-SBF, HP-SBF, HC-SBF, 960 mL) and the agarose sol containing apatite nuclei were mixed and dispersed by ultrasonic vibration again at 38.0 °C. The mixed solution was rotated in an incubator at 36.5 °C.

Formed hydroxyapatite microcapsules were collected by filtration using a 300 nm nitrocellulose membrane filter (MF-Millipore, Millipore Corporation, USA). The obtained microcapsules were observed by scanning electron microscopy (SEM; SU6600, Hitachi High-Technologies, Japan) and analyzed by energy dispersive X-ray analysis (EDX; XFlash 5010, Bruker, Germany) and X-ray diffraction (XRD; the Ultima IV, Rigaku, Japan) with Cu-K α radiation.

The decrement of Ca and P concentrations associated with formation of hydroxyapatite microcapsules in HC-SBF was measured by inductively coupled plasma atomic emission spectroscopy (ICP; ICP-7510, Shimadzu, Japan).

3. RESULTS AND DISCUSSION

3.1. The formation of microcapsules in NM-SBF

By using NM-SBF, the hydroxyapatite microcapsules were formed within 7 days. FIGURE 1 (a) shows that the SEM micrograph of the hydroxyapatite microcapsules fabricated in NM-SBF at low magnification. A number of spherical microcapsules were observed. FIGURE 1 (b) shows that at high magnification. It was observed that the microcapsule was constructed of flake-like crystallites which characterize bone-like apatite. It is considered that the apatite crystals were covered with the microparticles of agarose gels. FIGURE 1 (c) shows the result of EDX analysis of hydroxyapatite microcapsule. Peaks of Ca and P, the constituents of the hydroxyapatite, were observed.

The results indicate that an agarose gel containing apatite nuclei induced hydroxyapatite and formed microcapsules within 7 days by using NM-SBF.

FIGURE 1 (a) The SEM micrograph of microcapsules by soaking in NM-SBF for 7 days at low magnification.

FIGURE 1 (b) The SEM micrograph of a microcapsule by soaking in NM-SBF for 7 days at high magnification.

FIGURE 1 (c) The result of EDX analysis of hydroxyapatite microcapsules by soaking in NM-SBF for 7 days.

3.2. The formation of microcapsules in HP-SBF

By using HP-SBF, the microcapsules were formed within 2 days. In the SEM study, FIGURE 2 (a) and (b) were obtained. Spherical hydroxyapatite microcapsules were observed. FIGURE 2 (c) shows the result of EDX. Peaks of Ca and P were observed. The obtained results were similar to the 3.1. An agarose gel containing apatite nuclei induced hydroxyapatite in HP-SBF and formed microcapsules within 2 days.

These results indicate that raising pH of SBF promoted hydroxyapatite-forming reaction. The formation of hydroxyapatite in an aqueous solution can be described by the following equilibrium.

Consequently, ionic activity product (IP) of hydroxyapatite is given by the following formula where α is the activity, γ is the activity coefficient, and $[]$ is the concentration of each ion.

$$\begin{aligned} \text{IP} &= (\alpha\text{Ca}^{2+})^{10}(\alpha\text{PO}_4^{3-})^6(\alpha\text{OH}^-)^2 \\ &= (\gamma\text{Ca}^{2+})^{10}(\gamma\text{PO}_4^{3-})^6(\gamma\text{OH}^-)^2 \\ &\quad \times [\text{Ca}^{2+}]^{10}[\text{PO}_4^{3-}]^6[\text{OH}^-]^2 \end{aligned} \quad (2)$$

It is considered that IP was increased by raising $[\text{OH}^-]$ of SBF and the formation of microcapsules were accelerated.

FIGURE 2 (a) The SEM micrograph of microcapsules by soaking in HP-SBF for 2 days at low magnification.

FIGURE 2 (b) The SEM micrograph of a microcapsule by soaking in HP-SBF for 2 days at high magnification.

FIGURE 2 (c) The result of EDX analysis of hydroxyapatite microcapsules by soaking in HP-SBF for 2 days.

3.3. The formation of microcapsules in HC-SBF

By using HC-SBF, the microcapsules were formed within 2.5 hours. In the SEM study, FIGURE 3 (a) and (b) were obtained. FIGURE 3 (a) and (b) shows that hydroxyapatite microcapsules were observed. FIGURE 3 (c) shows that the result of EDX. Peaks of Ca and P were observed. The obtained results were similar to the 3.1. and 3.2. An agarose gel containing apatite nuclei induced hydroxyapatite in HC-SBF and formed microcapsules within 2.5 hours.

These results indicate that raising ion concentrations of SBF promoted hydroxyapatite formation. The results suggested that increase in $[Ca^{2+}]$ and $[PO_4^{3-}]$ caused the rise in IP by the Equations (1) and (2). Therefore, the degree of supersaturation against hydroxyapatite and ion

collision frequency was increased. As a result, hydroxyapatite formation was accelerated.

FIGURE 3 (a) The SEM micrograph of microcapsules by soaking in HC-SBF for 2.5 hours at low magnification.

FIGURE 3 (b) The SEM micrograph of a microcapsule by soaking in HC-SBF for 2.5 hours at high magnification.

FIGURE 3 (c) The result of EDX analysis of hydroxyapatite microcapsules by soaking in HC-SBF for 2.5 hours.

3.4. The XRD analysis of microcapsules by using each SBF

FIGURE 4 shows that the XRD patterns of the hydroxyapatite microcapsules by using NM-SBF, HP-SBF and HC-SBF. The blue squares represent the peak positions and intensity assigned to hydroxyapatite (JCPDS 24-0033). Not only the peak positions in the XRD pattern corresponded to hydroxyapatite, but broad peaks specific to bone-like apatite were observed for all conditions.

FIGURE 4 The XRD patterns of hydroxyapatite microcapsules by using each types of SBF.

3.5. The formation period in HC-SBF

FIGURE 5 shows that the concentration of Ca and P in HC-SBF during hydroxyapatite microcapsules formation. It is considered that the growth of hydroxyapatite microcapsules was almost completed for 3 hours. The concentrations hardly changed for the first hour. It appears that the formation of hydroxyapatite microcapsules were promoted in the SBF-hydroxyapatite microcapsules interface and therefore, the microcapsules were autocatalytically formed in the presence of the apatite nuclei. It is considered that the preferable formation period of hydroxyapatite microcapsules can be proposed from the results of ICP measurement.

4. CONCLUSION

The raising pH and ion concentrations of SBF accelerated the formation of hydroxyapatite microcapsules. The preferable formation period of hydroxyapatite microcapsules in HC-SBF was estimated at 3 hours by ICP measurement. It was

examined that the pH and the ion concentrations of SBFs affected the formation period of the hydroxyapatite microcapsules.

FIGURE 5 Changes in Ca and P concentrations in HC-SBF during hydroxyapatite formation.

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