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研究論文

Preparation of hydroxyapatite/calcium alginate composite gel and its release characteristics by pH

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ABSTRACT

Bone filling materials made from hydroxyapatite are used for treating bone defects caused by disease or injury. This material promotes the reproduction of new bone by injection into bone cavities. Even though hydroxyapatite has been utilized as a bone-filling material, it requires a long time to be replaced with host bone after implantation. Composite materials composed of hydroxyapatite and hydro-gels would be suitable to fill large bone defects and would be easily absorbed by the patient's body. Furthermore these materials could be used for drug carrier by stimulus response.

In this study, a hydroxyapatite/calcium alginate composite gel containing VB_{12} was synthesized in one step and the release characteristics of hydroxyapatite/calcium alginate composite gels were evaluated. The amount of VB_{12} released from the gels was maximum at pH 5 and the release of VB_{12} was inhibited at other than pH 5. The amount of VB_{12} released from the composite gel increased with the content of hydroxyapatite because hydroxyapatite made the pore size of the composite gel larger. Therefore the release characteristics of the composite gel could be controlled by pH and the content of hydroxyapatite. It is estimated that the composite gels containing drugs are expected to be applied to bone filling materials which have the function of drug carrier.

Key words: Hydroxyapatite, Calcium alginate, Composite gel, Release characteristics

1. Introduction

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is principal component of bone and teeth. Hydroxyapatite, which has a good biocompatibility and a high biological affinity, is used as bone-filling material for treating bone defects caused by disease or injury. This material promotes the reproduction of new bone by injection into bone cavities. Even though hydroxyapatite has been utilized as a bone-filling material, it requires a long time to be replaced with host bone after implantation [1-3].

In our previous work, hydroxyapatite was synthesized with a calcium chloride ($CaCl_2$) solution and a diammonium hydrogenphosphate ($(NH_4)_2HPO_4$) solution in a hydro-gel [4]. This material consisted of hydro gel as a soft material would be suitable to fill in a large bone defect and would adhere tightly to bone defect. Therefore this material would be rapidly assimilated into the patient's body.

Sodium alginate was used as hydro-gel. It forms polymer gel called calcium alginate by ion binding between carboxylate ion groups and Ca^{2+} ions [5, 6]. Calcium alginate is an ionic gel known as a material which contracts and swells by electric stimulation and pH. Calcium alginate gels containing drug are expected to have the function of a drug carrier [7,8].

Bone is a living tissue which is continually adapting its biological environment via continuous formation and resorption [9]. It is said that bone remodeling and this dynamic process occurs in both cortical and trabecular (or cancellous) bone allowing a rapid response to changes in circulating calcium levels [10, 11]. Bone formation occurs in which calcium was ingested into organic bone matrix secreted from osteoblasts [12]. Bone resorption occurs in which organic bone matrix was decomposed by enzyme from osteoclasts and inorganic bone matrix was decomposed by H^+ ion from osteoclasts [12]. Especially pH of resorption lacunae after bone resorption was decreased to 3-4 [13]. As described above, the pH is changed in *in vivo* bone during bone remodeling. Therefore, this composite gel is expected to be drug carrier which responses to the pH change *in vivo* bone.

We have also reported about the stimuli responsive characteristics of hydroxyapatite/calcium alginate composite gels [14]. The change in volume of the composite gels was related to pH. The change in volume of the composite gels and calcium alginate gels was dependent on the dissociation of carboxyl groups with the environmental solution. Additionally, the stretching behavior of the composite gels depended on the content of hydroxyapatite.

In this study, Vitamin B_{12} (VB_{12}) was used as a model of drug. VB_{12} is able to consume in our body. Its solution is red in color and concentration of VB_{12} in solution can be measured with UV-visible spectrophotometer. The composite gels containing VB_{12} were prepared and the amount of VB_{12} which had been released from the composite gels with alteration of pH was investigated.

2. Experimental

2.1 Materials and Reagents

Calcium chloride ($CaCl_2$) (FW=110.98), diammonium hydrogenphosphate ($(NH_4)_2HPO_4$) (FW=132.06), 0.01 M hydrochloric acid (N/100) (HCl, 0.04% hydrochloric acid in water) and 0.01 M sodium hydroxide solution (N/100) (NaOH, 0.04% sodium hydroxide in water) were obtained from Kanto Chemical Co., Inc. Sodium alginate (300 cps) was obtained from Nacalai Tesque, Inc. Cyanocobalamine (Vitamin $B_{12,}$ VB_{12}) ($C_{63}H_{88}CoN_{14}O_{14}P$) (MW=

1355.40) was obtained from Wako Pure Chemical Industries, Ltd. These agents were used without further purification.

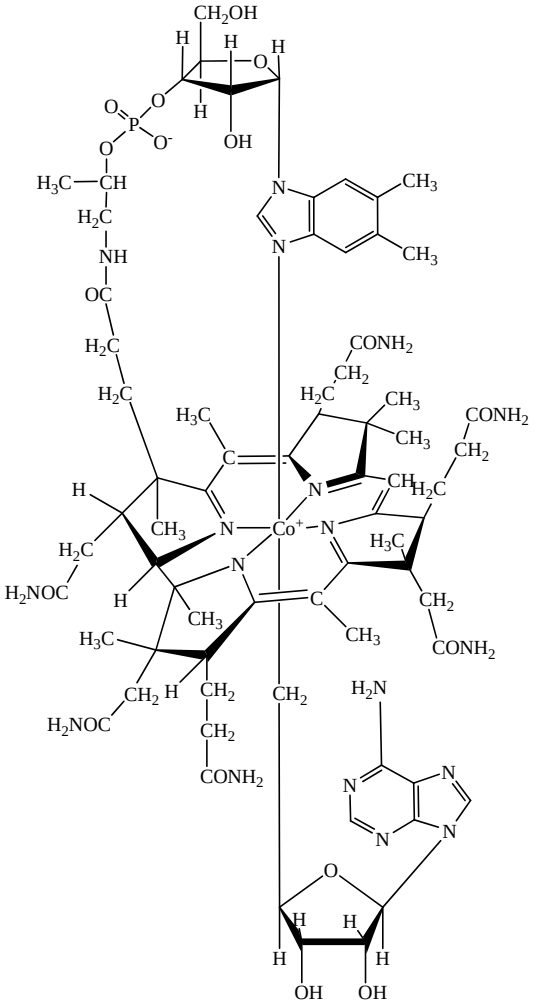

Figure 1 Structure of VB_{12}.

2.2 Preparation of hydroxyapatite/calcium alginate composite gel containing VB_{12}

0.1 mM VB_{12}-$(NH_4)_2HPO_4$ (120 mM and 480 mM)-1% sodium alginate aqueous solution were dropped into 0.1 mM VB_{12}-$CaCl_2$ (250 mM and 1000 mM) aqueous solution, which was immersed in VB_{12}-$CaCl_2$ aqueous solution until equilibrium state. Two types of composite gels containing 0.1 mM VB_{12}: 250 mM $CaCl_2$-120 mM $(NH_4)_2HPO_4$ -1% calcium alginate composite gels and 1000 mM $CaCl_2$-480 mM $(NH_4)_2HPO_4$ -1% calcium alginate composite gels were synthesized. In addition, calcium alginate gels containing VB_{12} were also prepared with 1% sodium alginate solution and 100 mM $CaCl_2$ solution without $(NH_4)_2HPO_4$ present. The gels were observed with stereomicroscope (Shimadzu Manufacturing Co., Ltd., STZ-168-BL/TL).

2.3 Observation of pH dependence of composite gels

Composite gels and calcium alginate gels containing VB_{12} were immersed in distilled water for 0.5-5 hours. The gels were sliced to 1 mm thickness with a razor and the sliced gels were observed with stereomicroscope (Shimadzu Manufacturing Co., Ltd., STZ-168-BL/TL). The experiment was carried out with three types of gels (1% calcium alginate, 250 mM $CaCl_2$-120 mM $(NH_4)_2HPO_4$-1% calcium alginate and 1000 mM $CaCl_2$-480 mM $(NH_4)_2HPO_4$-1% calcium alginate) and using 0.01 M HCl or 0.01 M NaOH solutions instead of distilled water.

2.4 Evaluation of release characteristics of composite gels

0.01 M HCl solution has pH 2, distilled water has pH 5 and 0.01 M NaOH solution has pH 10. The pH 2-10 solutions were prepared with 0.01 M HCl solution and 0.01 M NaOH solution. 10 beads of gels containing VB_{12} were immersed in 5 mL of each solution for 1.0-5.0 hours and the absorbance of these solutions was determined with UV-visible spectrophotometer. Then amount of released VB_{12} was calculated by calibration curve method. In addition, the pore diameter of the gel was measured with SEM images of the cross-section of

the gels.

3 Results and discussion

3.1 Preparation of hydroxyapatite/calcium alginate composite gel containing VB_{12}

It was reported that the composite gels were prepared with $CaCl_2$ solution and $(NH_4)_2HPO_4$-sodium alginate solution [4]. The composite gels were white in color and spherical in shape. Hydroxyapatite was successfully generated with $CaCl_2$ and $(NH_4)_2HPO_4$ in calcium alginate gel. The content of hydroxyapatite in the composite gel was measured with thermogravimetric analyzer, and 49.9% and 61.8% hydroxyapatite was contained in the composite gel prepared from 250 mM $CaCl_2$ and 120 mM $(NH_4)_2HPO_4$ and 1000 mM $CaCl_2$ and 480 mM $(NH_4)_2HPO_4$, respectively [4]. The content of hydroxyapatite in the gel was significantly related to the concentration of $CaCl_2$ and $(NH_4)_2HPO_4$.

In this study, the composite gels containing VB_{12} were easily prepared from $CaCl_2$ solution containing VB_{12} and $(NH_4)_2HPO_4$-1% sodium alginate solution containing VB_{12}.

The gels containing VB_{12} were red in color and spherical in shape (Figure 2). The size of the composite gels was larger than that of 1% calcium alginate gels. The size of the gels was depended on the content of hydroxyapatite.

3.2 Observation of pH dependence of composite gels

The alginate gel and the hydroxyapatite/calcium alginate gel decreased in volume in an acidic solution of pH 2. The gels containing more hydroxyapatite decreased in volume more slowly than the gels containing less hydroxyapatite. All gels increased in volume in the alkaline solution of pH 10.

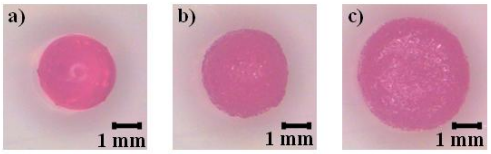

Figure 2 Photographs of the gel containing VB_{12}. a) 1% calcium alginate, b) 250 mM $CaCl_2$-120 mM $(NH_4)_2HPO_4$-1% calcium alginate and c) 1000 mM $CaCl_2$-480 mM $(NH_4)_2HPO_4$-1% calcium alginate.

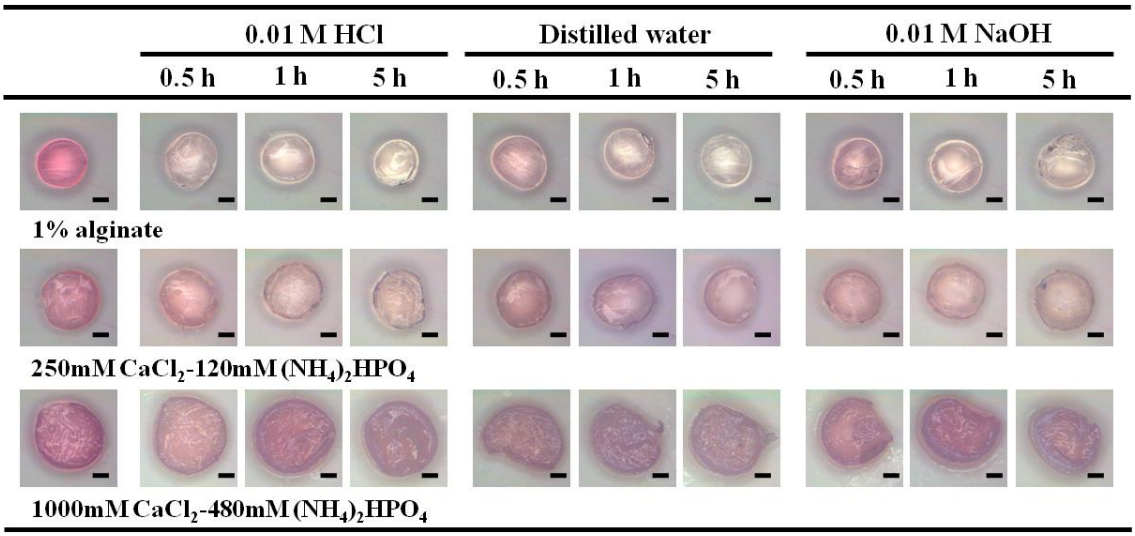

Figure 3 Photographs of the gels containing vitamin B_{12} immersed in the each solution for 0.5-5 hours. The length of the scale bars is equals to 1 mm.

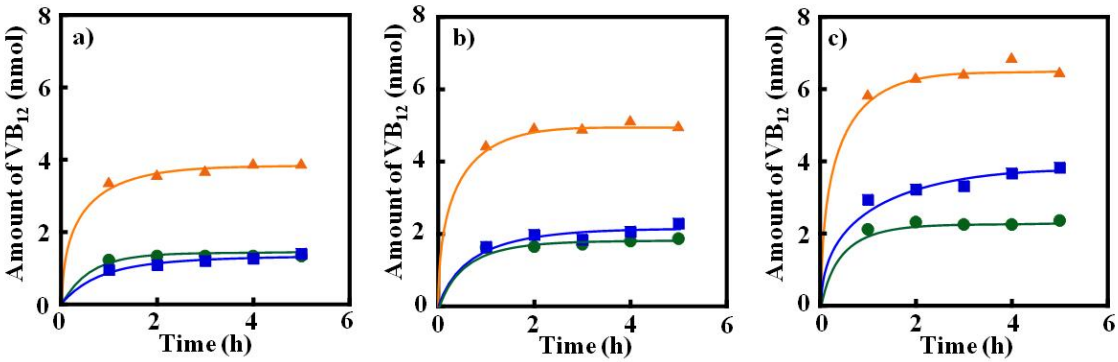

Figure 4 The graph of a) indicates 1% calcium alginate gels, b) indicates 250 mM $CaCl_2$-120 mM $(NH_4)_2HPO_4$ 1% alginate gels and c) indicates 1000 mM $CaCl_2$-480 mM $(NH_4)_2HPO_4$ 1% alginate gels. (■) the gels immersed in pH 10 solution, (▲), the gels immersed in pH 5 solution, (●) the gels immersed in pH 2 solution.

After the immersion, the gels sliced in about 1 mm thickness were observed with stereomicroscope (Figure 3). The color of the gels immersed in each solution faded away with time. Cross-section of the gels colored evenly from center to exterior. It is indicated that VB_{12} from the inside of the gels was diffused rapidly.

3.3 Evaluation of release characteristics of composite gels

Figure 4 shows amount of VB_{12} released from the gel with time due to pH. The release of VB_{12} from a gel was calculated. The amount of released VB_{12} in the case of calcium alginate gel was lower than that of composite gels. And the amount of VB_{12} in the case of the composite gel which had large content of hydroxyapatite was higher than that of the composite gel which had low content of hydroxyapatite. The amount of VB_{12} released from the gel had a high content of hydroxyapatite. Additionally, less amounts of VB_{12} were released from the gels immersed in solutions of pH 2 and 10 than that of pH 5.

The pH dependence of release characteristic was evaluated (Figure 5). The amount of VB_{12} released from the gels was maximum at pH 5 and the release of

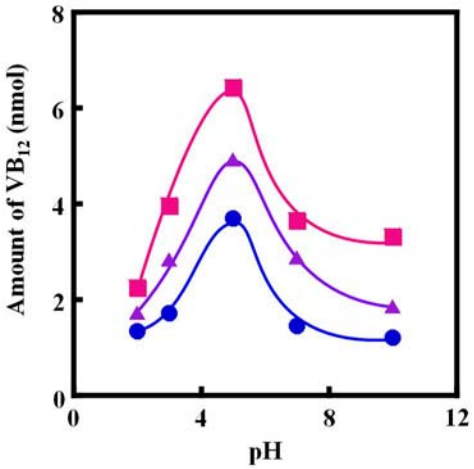

Figure 5 pH dependence of release characteristic. (■) 1000 mM $CaCl_2$-480 mM $(NH_4)_2HPO_4$ 1% alginate gels, (▲) 250 mM $CaCl_2$-120 mM $(NH_4)_2HPO_4$ 1% alginate gels, (●) 1% calcium alginate gels.

Table 1 Release speed of the gels immersed in each solution at one hour

	Release speed (nmol/min)		
	pH 2	pH 5	pH 10
1% calcium alginate gel	0.20	0.56	0.16
250 mM $CaCl_2$ 120 mM $(NH_4)_2HPO_4$	0.27	0.74	0.27
1000 mM $CaCl_2$ 480 mM $(NH_4)_2HPO_4$	0.35	0.98	0.49

VB_{12} was inhibited at other than pH 5.

Release speed of the gels immersed in each solution at one hour was calculated (Table 1). The release speed of the gels at pH 5 was largest and that of the gels containing much of hydroxyapatite was largest of three types of the gels.

The pore diameter of the gels was measured on SEM images (Table 2). The pore size of the composite gels which have large content of hydroxyapatite, small content of hydroxyapatite, no hydroxyapatite were 0.7μm, 0.4 μm, less than 0.4 μm respectively. It is considered that hydroxyapatite increased the pore size and surface area of the composite gels as reaction field was increased. Therefore much of VB_{12} was easy to be released from the composite gels due to rapid diffusion of VB_{12}.

Table 2 Pore size of the composite gels.

	Diameter of pore (μm)
1% calcium alginate gel	Less than 0.4
250 mM $CaCl_2$ 120 mM $(NH_4)_2HPO_4$	0.4
1000 mM $CaCl_2$ 480 mM $(NH_4)_2HPO_4$	0.7

Figure 6 shows Mechanism of release characteristics due to pH. In pH 5 solution, the size of the network of the gels was expanded. VB_{12} could easily pass through the network of the gels. In the solution of pH 2, the size of the network was constricted and a skin layer appeared [15]. The VB_{12} were difficult to pass through the network of the gels. In the solution of pH 10, the network of the gel was dilated. However, the release of VB_{12} was inhibited. VB_{12} are charged positively for its structure (Figure 1) and carboxyl groups of alginate were dissociated into carboxylate anion; therefore the VB_{12} were trapped by the network.

From these results, the amount of VB_{12} depended on the pH of the solution and the content of hydroxyapatite.

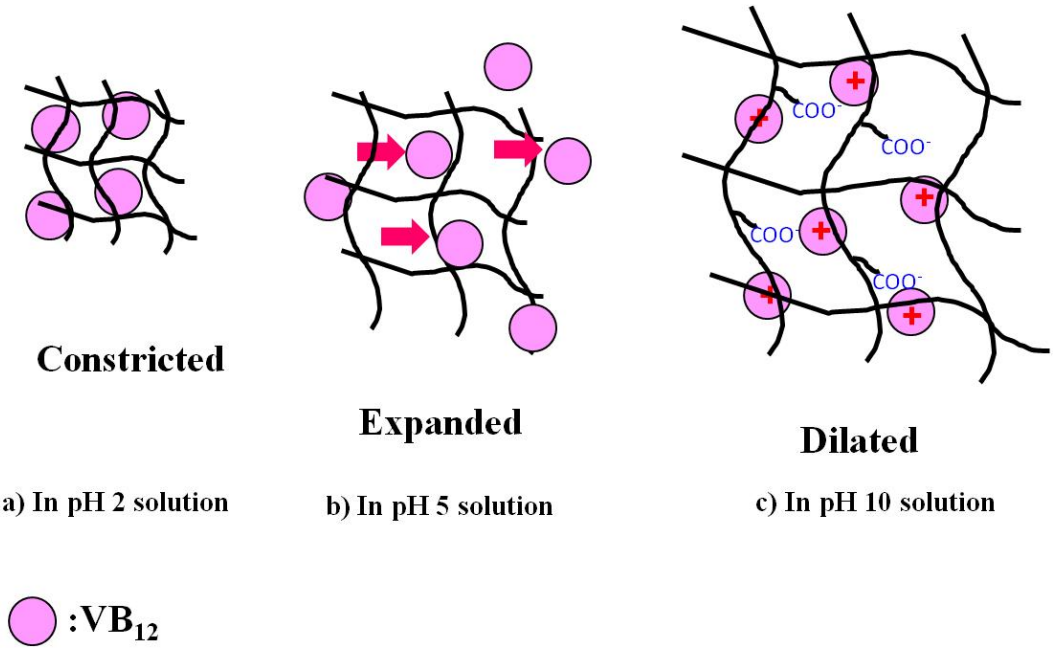

Figure 6 Mechanism of release characteristics due to pH. The gels immersed in a) pH 2 solution, b) pH 5 solution and c) pH 10 solution.

It is estimated that the amount of released VB_{12} can be controlled by pH and the content of hydroxyapatite.

4 Conclusions

A hydroxyapatite/calcium alginate composite gel containing VB_{12} was synthesized in one step from $(NH_4)_2HPO_4$–sodium alginate solution containing VB_{12} and $CaCl_2$ solution containing VB_{12}. The amount of VB_{12} released from the gels was largest at pH 5 and the release of VB_{12} was inhibited at other than pH 5. In addition, the amount of VB_{12} released from the composite gels containing much of hydroxyapatite was higher than that of the composite gels containing less hydroxyapatite. VB_{12} in the composite gels containing much hydroxyapatite were easy to diffuse because the pore size of the gel was large. Therefore the release characteristics of composite gel can be controlled by pH and the content of hydroxyapatite. It is estimated that composite gels containing drug are expected to be applied to bone filling materials which have the function as a drug carrier.

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ハイドロキシアパタイト／アルギン酸複合ゲルの作製と薬物徐放特性

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ハイドロキシアパタイトは骨や歯の主成分であり、骨補填材として利用されている。骨補填材とは骨内の空洞を補填し、骨組織と結合することで、新生骨の再生を促す材料である。ハイドロキシアパタイトは、生体親和性、生体適合性には優れているが、自家骨との置換に時間を要し、治癒の期間が長くなるなどの問題が生じている。そこで、柔軟な材料であるゲルを用いることで骨欠損部分の形状に合致し、生体内での分解・吸収性に優れた骨補填材を作製できると考えられる。本研究では、これまでにハイドロキシアパタイト／アルギン酸カルシウム複合ゲルを作製し、複合ゲルの電場や pH などの刺激応答特性を報告している。外部刺激やハイドロキシアパタイトの含有率により、体積変化率が変化することから、本報告では、複合ゲルにドラッグキャリアとしての機能を付与するため、pH 応答による物質放出特性について基礎的知見を得たので報告する。

キーワード：ハイドロキシアパタイト、アルギン酸カルシウム、複合ゲル、放出特性