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Protein release parameters estimated with a flow system on zinc-containing apatite

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Abstract. Adsorption and desorption properties of proteins on zinc-containing apatite were successively monitored with a newly-developed flow system, and sustained-release ability of the apatite with different zinc contents was evaluated using protein release parameters we suggested. Three sustained-release parameters; initial desorption rate (r_{init}), time of desorption-completed (T_{des}), and desorption constant (k_d) were estimated with graphical analysis of dynamic desorption curves in a flow of 20 mM phosphate-buffered solution (PBS). Bovine serum albumin (BSA) of isoelectric point (*pI*) 4.8 and egg white lysozyme (LSZ) of *pI* 11.2 were employed as model protein drugs. Incorporation of zinc into hydroxyapatite changed desorption responses of the proteins. Zn(0.15), where the number in parentheses denoted the preparing molar ratio of Zn/Ca, showed the most sustained-release ability: less r_{init}, longer T_{des}, and smaller k_d. Furthermore, the adsorbed amounts of the proteins for Zn(0.15) were 1.5 ~ 4 times larger than Zn(0), which suggested that Zn(0.15) would be promising as a sustained-release carrier of protein drugs.

1. Introduction

Hydroxyapatite (HAp), which is a primary inorganic component of bone and tooth, has been recently paid attention as a sustained-release carrier of protein drug [1, 2, 3, 4]. HAp has two advantageous properties as the protein carrier; one is biocompatibility and therefore that does not cause inflammation to tissues. The other is hydrophilicity, making it possible to manufacture protein drugs in water, thus preventing them from denaturation and deactivation.

Zinc has been known as a trace element of the human body, and is contained in more than 300 enzymes and works as Lewis acid. Furthermore, zinc forms stable globular domains of protein by bridging amino residues such as cysteine and histidine. These domains are called Zinc Finger, which are involved in transcription and translation of genes [5, 6, 7]. Because of this function of stabilizing protein structure, sustained-release drugs containing zinc have been developed [8]. Zinc-doped HAp was also suggested to be a promising sustained-release drug career, taking both the advantages of zinc and HAp [9, 10, 11, 12, 13]. However, information on the sustained-release ability of this material seems to be insufficient, especially the dependency of zinc content on the sustained-release properties.

Furthermore, quantitative parameters would be necessary for evaluating sustained-release ability in *in vitro* approach as a preliminary to *in vivo* examination.

We developed a new flow system to monitor successively adsorption and desorption properties of protein on solid material in order to evaluate sustained-release ability more easily and clearly than a batch system, because readsorption was observed in the batch system, making the desorption properties ambiguous [14]. With the new flow system, the desorption response was able to be clearly monitored possibly because the desorbed protein flew off the material surface immediately. Furthermore, the adsorbed and desorbed amount of the protein and the desorption ratio: (r_{de}); the ratio of desorbed amount/initially adsorbed one, were able to be estimated in one experiment.

However, additional parameters would be necessary to evaluate sustained-release ability more quantitatively as mentioned before. In this manuscript, three sustained-release parameters; initial desorption rate (r_{init}), time of desorption-completed (T_{des}), and desorption constant (k_d) were estimated with graphical analysis of the desorption curve. The dependency of zinc content of HAp on the three parameters, the r_{de}, and the adsorbed amount was examined.

2. Materials and methods

2.1. Preparation of the samples

Zn-containing HAp was prepared by a wet synthesis method. Aqueous solution (aq. sol.) of mixture of 0.1 M calcium nitrate four-hydrate {$Ca(NO_3)_2 \bullet 4H_2O$} and given molar zinc nitrate six-hydrate {$Zn(NO_3)_2 \bullet 6H_2O$} was flown into 0.06 M diammonium hydrogen phosphate {$(NH_4)_2HPO_4$} aq. sol. at a rate of 4 cm^3/min at 323 K under pH 7. The obtained slurry was dried and put in an electric oven at 373 K overnight. The powder thus prepared was used as a sample. The preparing molar ratio of Zn/Ca was 0 ~ 0.2, where the sample is expressed as Zn(0), Zn(0.1), Zn(0.15), or Zn(0.2) according to the molar ratio.

2.2. Adsorption and desorption behavior of protein

Bovine serum albumin (BSA, Sigma-Aldrich A4503) and egg white lysozyme (LSZ, Sigma-Aldrich L6876) were employed as model protein drugs. The isoelectric points (*pI*) of BSA and LSZ were 4.8 and 11.2, respectively.

Adsorption and desorption response of protein was continuously monitored with a customized commercial equipment: ATTO Biochromatograph II. This apparatus is composed of a control unit attached to six electric valves for automatic switching of solution, a monitor, a sample-packed column, a peristaltic pump to introduce the solution into the column, a UV detector, and a recorder. 0.5 g sample powder of was packed into a layer 1.25 cm thick. The adsorption and desorption responses of protein was followed as a function of time, in flowing 1.0 g protein$\cdot dm^{-3}$ - 5 mM phosphate-buffered solution (PBS) and successively switching to 5 mM PBS and 20 mM PBS as the eluents.

2.3. Protein release parameters

Curve 1 in figure 1 shows an imaged adsorption and desorption curve for the sample-loaded column. The curve was redrawn in order to eliminate the time lag in switching the solutions; the time lag to be subtracted from the original response time was 2 min 20 sec. Furthermore, the effect on the curve of the time to replace one solution by another in the column was corrected by the graphical difference between the response for the sample-loaded column and that for a blank column, in which styrene-divinylbenzene copolymer beads (20% linked) (Strem Chemicals Co.) was loaded (shown by Curve 2 in figure 1). Therefore, the adsorbed and desorbed amounts of protein were estimated by graphical integration of the shaded areas in figure 1, the former being the lag area up to the plateau of the curve in protein-PBS flow (A), and the latter being the lag area down to zero in the PBS flow (B for 5 mM PBS and C for 20 mM PBS respectively).

Three sustained-release parameters; initial desorption rate (r_{init}: mg•cm^{-3}•hr^{-1}), time of desorption-completed (T_{des}: hr), and desorption constant (k_d: hr^{-1}) were estimated with graphical analysis of a desorption curve in the flow of 20 mM PBS, where better release properties can be considered to lead to less r_{ini}, longer T_{des}, and smaller k_d.

k_d was estimated as follows: assuming to be the first order kinetics, desorption rate (mg·cm^{-3}·hr^{-1}) can be expressed as

$$- d\,Q_{rem} / d\,t = k_d \bullet Q_{rem} \quad \bullet\bullet\bullet \text{ (i)}$$

where Q_{rem} is the adsorbed amount remaining at time = t (mg•cm^{-3}). As desorption ratio X_t at t is expressed as $(Q_{total} - Q_{rem}) / Q_{total}$, where Q_{total} is the total desorbed amount, eq. (i) is converted to

$$d\,X_t / d\,t = k_d\,(1 - X_t) \quad \bullet\bullet\bullet \text{ (ii)}$$

Integrating eq. (ii),

$$\ln (1 - X_t)^{-1} = k_d \bullet t \quad \bullet\bullet\bullet \text{ (iii)}$$

Plotting $\ln (1 - X_t)^{-1}$ vs. t gives a straight line, and so k_d can be obtained by its slope.

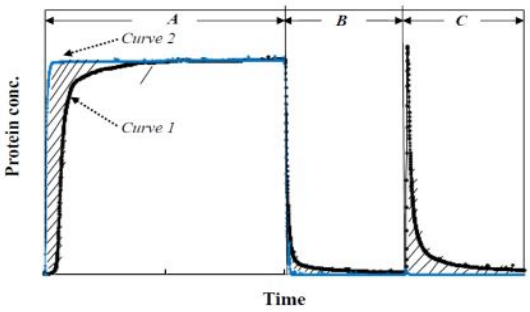

Figure 1 Image of adsorption and desorption response of protein on apatite powder with a flow system.
A 1.0 g-protein·dm^{-3} - 5 mM PBS; B 5 mM PBS; C 20 mM PBS; Curve 1 apatite loaded; Curve 2 polystyrene-beads loaded as a blank; loaded amount 0.5 g; flow rate 1 cm^3·min^{-1}.

3. Results and discussion

3.1. Characterization of the samples

From the XRD patterns of the samples, the single phase of apatite structure was observed for Zn (0), Zn (0.1), and Zn (0.15). For Zn(0.2), $CaZn_2(PO_4)_2$•$2H_2O$: scholzite was observed in addition to apatite with low crystallinity. This result was also suggested by Miyaji *et al.* [15]. Furthermore, they concluded that the limit of substituting ratio of Zn to Ca was 15 mol %, which is close to Zn (0.15) in the present sample, for which the estimated ratio was 14.8 mol% from our chemical analysis.

3.2. Adsorption and desorption behavior

The adsorbed amounts of BSA and LSZ were compared for the four samples in figure 2 (a) and (b), where the left open bar and the right filled one were the amount per mass and that per surface area, respectively for each sample. Zn(0.15) had much the most amounts of both proteins, while Zn(0.1) showed not so much difference in the amounts from Zn(0). This may indicate that Zn with the limit content in apatite structure contributed to the increase of the amounts.

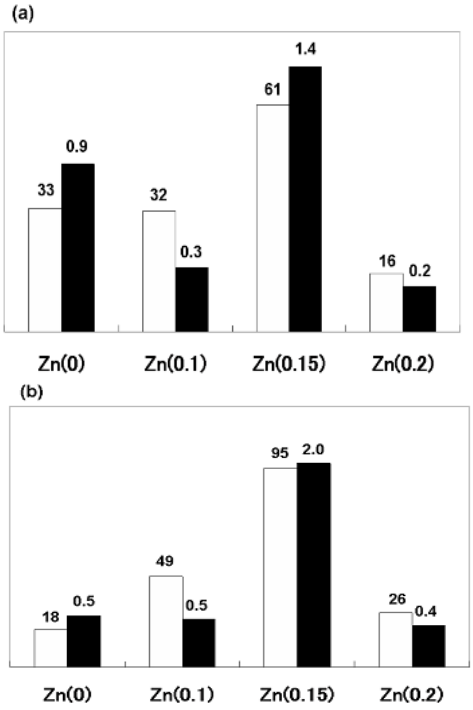

Figure 2 Adsorbed amounts of BSA (a) and LSZ (b). □ mg-BSA•g^{-1}; ■ mg-BSA•m^{-2}

Three sustained-release parameters: initial desorption rate (r_{init}), time of desorption-completed (T_{des}), and desorption constant (k_d) were estimated from the graphical analysis of the desorption curves in 20 mM PBS. These parameters were compared in figure 3(a) for BSA and (b) for LSZ, respectively. For BSA (a), r_{init} decreased as the increase of zinc content. For other two parameters, Zn (0.15) showed the best sustained-release properties of all the samples; the smallest k_d and the longest T_{des}, where the desorption did not complete within twelve hours: our programmed-time. For LSZ (b), zinc did not contribute to the decrease of r_{init} while T_{des} and k_d drastically changed by the presence of zinc. T_{des}

became one-order larger and k_d one-order smaller for Zn (0.1), Zn (0.15), and Zn (0.2) than Zn (0). Among the zinc-containing apatites, Zn (0.15) was shown to be a promising sustained-release carrier, especially of BSA, considering much more adsorbed amounts as well as the values of the three sustained-release parameters.

With our new experimental and analytical approach, sustained-release ability of protein was able to be evaluated for the zinc-containing apatite. This *in vitro* system is considered to be available for obtaining preliminary information on possibility of solid materials as sustained-release carrier prior to *in vivo* approaches.

4. Conclusions

Three sustained-release parameters of protein drug were suggested with a newly-customized flow system. Sustained-release properties of BSA on hydroxyapatite were drastically improved; less *initial desorption rate*, longer *time of desorption-completed*, and smaller *desorption constant* with a large increase of the adsorbed amounts by substituting Zn for Ca by 15 mol %.

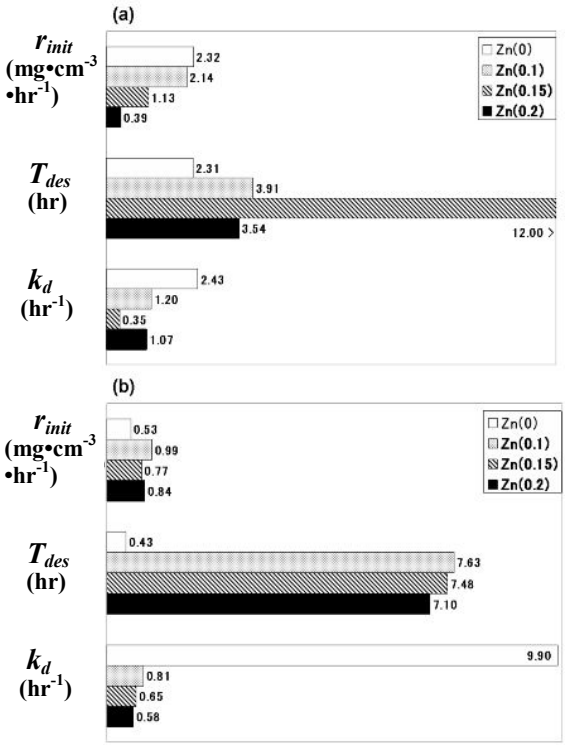

Figure 3 Sustained-release parameters for BSA (a) and LSZ (b).

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