

Effect of pH on the Formation of Lysosome-Alginate Beads for Antimicrobial Activity

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In this study, we developed lysosome-alginate beads for application as an oral drug delivery system (ODDS). The beads harboring lysosomes, which have antimicrobial activity, and various concentrations of alginate were characterized and optimized. For application as an ODDS, pH-dependent lysosome-alginate beads were generated, and the level of lysosome release was investigated by using antimicrobial tests. At low pH, lysosomes were not released from the lysosome-alginate beads; however, at neutral pH, similar to the pH in the intestine, lysosome release was confirmed, as determined by a high antimicrobial activity. This study shows the potential of such an ODDS for the *in vivo* treatment of infection with pathogens.

Keywords: Lysosomes, alginate, oral drug delivery system

In recent years, biopolymer use in the design of biodegradable particles as delivery systems on the nanometer- to micrometer-length scale has received much attention [5]. Alginate is an anionic biopolymer containing 1,4-linked α -mannuronic and β -guluronic acid residues [1]. Alginate, which is a sodium salt of alginic acid and a biodegradable and mucoadhesive natural polymer, has been widely used as a delivery vehicle for the controlled release of therapeutic agents [8, 9]. The capacity of alginate to form spherical gel beads in the presence of multivalent cations (ionotropic gelation technique) has been explored to prepare multi-particulate systems incorporating numerous drugs, proteins, cells, or enzymes. When administered orally, the release of drugs from alginate beads is pH controlled and occurs in the intestine, mainly by diffusion through the swelled matrix owing to an erosion mechanism [3, 12]. The biopharmaceutical characteristics of alginate beads prepared by ionotropic gelation depend on

their size and shape as well as their morphology [6, 7]. It has been demonstrated that optimization of the bead shape, size, and morphology to generate uniform beads can be achieved by altering the processing parameters of the electrospray method.

Lysosomes are intracellular organelles containing 50 to 60 hydrolases and represent the cellular site for bulk macromolecule degradation. The activities of lysosomes mediate several processes in cell feeding and antimicrobial defense, which involve lysosome fusion with endosomes and autophagosomes. We previously observed the *in vitro* activity of lysosomes after extraction from egg white or certain eukaryotic cells [12]. In the present study, we focused on the whole lysosome's activity to maintain specific antimicrobial activity after preparing mixed beads composed of lysosomes and alginate. Thus, we used the lysosome as a model drug from hen's egg white, which possesses many biologically active proteins that could offer

better valorization of hen's egg white. Lysozyme can be obtained from hen's egg white, which contains 54% albumin, 13% ovotransferrin, 11% ovomucoid, and 3.4% lysozyme [10, 11], with antimicrobial, antiviral, anti-inflammatory, and analgesic effects [4], respectively. Additionally, ovotransferrin is an antimicrobial agent; avidin is a vitamin carrier and antimicrobial agent; flavoprotein is an antihypertensive agent; and ovomucin is a source of glycopeptides with antiviral, antitumor, and immunomodulatory effects [4]. Therefore, we studied lysosome-alginate beads as an oral delivery system for the *in vivo* treatment of infection with pathogens. These beads were expected to overcome the usual loss of drug activity owing to the retention time in the stomach at low pH, thereby providing effective oral delivery of drugs.

Escherichia coli K-12 was used for antimicrobial activity tests. The preparation of lysosomes from egg white and the antimicrobial activity tests were performed as previously reported [13]. Briefly, 5 ml of 2% sodium alginate (Sigma-Aldrich) in sterilized distilled water was prepared. Additionally, 2% (w/v) calcium chloride was prepared at various pH values, in the range of pH 3 to 6, for the observation of lysosome release on exposure to HCl. Various concentrations of lysosomes (~10% to 50%) were mixed with 0.1 g of sodium alginate dissolved in sterilized distilled water by vortexing. Spherical bead droplets were generated by extrusion of the solution containing sodium alginate mixed with lysosomes through a 30-gauge needle and a 50 cm sterilized silicone tube (Cole-Parmer) using a syringe pump (KD Scientific) equipped with a 10 ml disposable plastic syringe (Ormond Beach). The suspension of lysosomes and sodium alginate was forced out of the tip of the needle at a constant flow rate of 33.3 μ l/min, and the droplets formed under both electrostatic and gravitational forces. The electrostatic potential was generated at 10 kV by connecting the negative electrode of a high-voltage DC unit (Model ES30P 10 W, HV power supply; Gamma High Voltage, FL, USA) to the needle and the positive aluminum electrode to the grounding gelling bath, which was 100 ml of sterilized 2% (w/v) CaCl₂ (Sigma-Aldrich) solution. The negative electrode/needle tip was positioned 7 cm above the surface of the hardening solution. After the formation of the beads, they were stirred in the 2% (w/v) CaCl₂ for hardening for 60 min to ensure completion of the gelling process. The beads were then rinsed, washed with LB medium, and stored in 50 ml centrifuge tubes to maintain humidity in LB medium at 4°C until use. This bead generation was repeated at different pH values of calcium chloride solution to investigate the stability of the lysosome-alginate

beads. To check the surface differences of the lysosome-alginate beads under different pH conditions, scanning electron microscopy (SEM) was used. The lysosome-alginate beads were fixed and dried. The dried samples were coated with gold under reduced pressure, and surface images were recorded using an FE-SEM (S-4800; Hitachi, Japan).

The electrospray method was used to generate lysosome-alginate beads of uniform size. To investigate the effect of bead formation on antimicrobial activity, five concentrations of lysosomes (10%, 20%, 30%, 40%, and 50%) and three alginate concentrations (1%, 2%, and 3%) were prepared and combined to generate different sets of beads. While electrospraying, the electrode distance from the hardening solution to the needle, the infusion rate, and the applied potential were fixed at 7 cm, 33.3 μ l/min, and 10 kV, respectively. Moreover, the pH of the 2% calcium chloride was fixed at a neutral pH of 6.8. As shown in Table 1, 1% alginate with a high concentration of lysosomes (40% or 50%) could not form beads. A 1% alginate solution might not allow the formation of beads because of loss of the solution's viscosity at a 40% or 50% lysosome concentration. The other combinations showed stable bead formation, with an antimicrobial effect on *E. coli*. Based on the antimicrobial activity, a combination of 20% lysosomes with 2% alginate was optimal. Therefore, 2% alginate was used for encapsulation in subsequent experiments. To investigate the antimicrobial activity of 2% alginate combined with various lysosome concentrations, we performed time-course experiments. In these experiments, the antimicrobial activity was examined at every 1 h for 5 h. From 0 to 120 min, all of the concentrations of lysosome-alginate beads did not show any significant antimicrobial activity against *E. coli* (data not shown). The antimicrobial activity of the lysosome-alginate beads began at 180 min, and at this point, the beads containing 20% lysosomes showed the greatest effect (~45% cell mortality) (Fig. 1). At 240 min,

Table 1. Summary of characterization of lysosome-alginate bead at different lysosome and alginate concentrations (+: 20 ~ 40; ++: 40 ~ 60; +++: 60 ~ 80).

Alginate conc.	Lysosome conc.	10%		20%		30%		40%		50%	
		A ^a	B ^b	A	B	A	B	A	B	A	B
1%		○	+	○	++	○	+	-	-	-	-
2%		○	++	○	+++	○	++	○	+	○	+
3%		○	+	○	+	○	+	○	+	○	+

^aBead formation.

^bAntimicrobial activity.

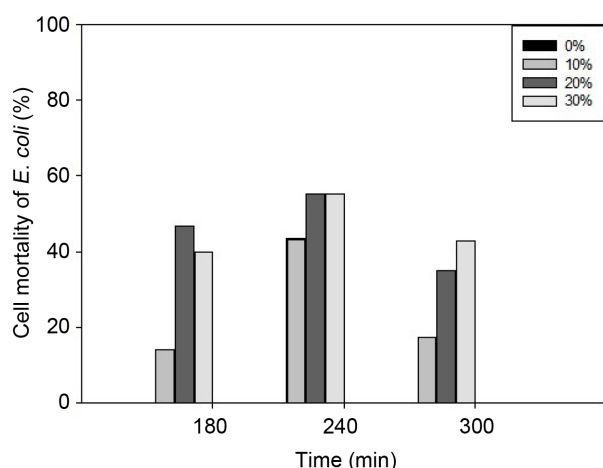


Fig. 1. Antimicrobial activity of alginate-lysosome beads against *E. coli*.

The beads were composed of 2% alginate mixed with a 10%, 20%, or 30% lysosome concentration. *E. coli* were treated with the alginate-lysosome beads for 180, 240, or 300 min. Here, 0% means only 2% alginate beads, without lysosomes.

beads with all three lysosome concentrations showed an ~50% antimicrobial effect. Moreover, a 20% and a 30% lysosome content resulted in an ~60% cell mortality. Based on this result, we assumed that lysosomes were released from the encapsulated alginate beads at 180 min after exposure to *E. coli* and that the antimicrobial activity is maximal after 240 min. Therefore, further antimicrobial activity experiments were conducted after 240 min exposure to *E. coli*. Although 2% alginate beads containing 20% lysosomes showed significant antimicrobial activity, lysosome-alginate beads were subsequently generated under different

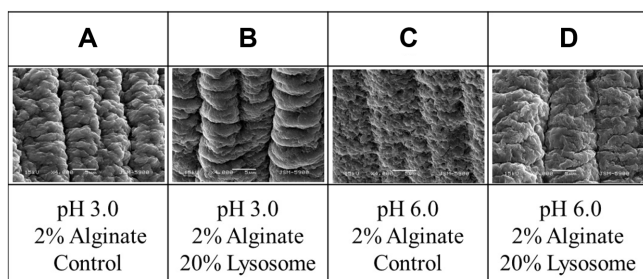


Fig. 2. Scanning electron microscopy image of 2% alginate and 20% lysosome-alginate beads generated under two pH conditions.

(A) 2% alginate beads formed at pH 3.0, without lysosomes. (B) Mixed beads consisting of 2% alginate and 20% lysosomes, formed at pH 3.0. (C) 2% alginate beads formed at pH 6.0, without lysosomes. (D) Mixed beads consisting of 2% alginate and 20% lysosomes formed at pH 6.0.

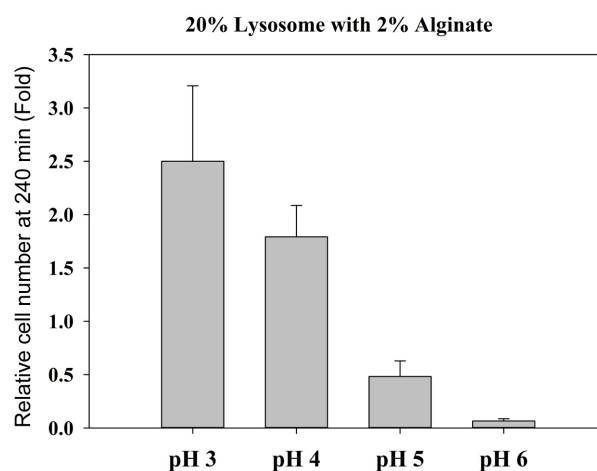


Fig. 3. Antimicrobial activity of beads composed of 2% alginate and 20% lysosome formed at pH 3, 4, 5, or 7.

Here, *E. coli* was used to confirm the antimicrobial activity, and the treatment time was 240 min.

pH conditions, and the release of lysosomes from these beads was investigated. Four types of lysosome-alginate beads were generated at different pH values, ranging from pH 3 to 6. At first, the effect of pH on lysosome-alginate bead formation was analyzed by observation of the morphology by SEM (Fig. 2). There were no morphological differences between 2% alginate beads and lysosome-alginate beads or between beads generated at pH 3 and pH 6. Next, lysosome release was determined by measuring the antimicrobial activity against *E. coli*. The lysosomes released from the beads showed ~45% antimicrobial activity at pH 6. However, the antimicrobial activity at the lower pH was difficult to observe, indicating that lysosomes were not released. Moreover, the cell number at 240 min after the exposure of *E. coli* showed a significant decrease at pH 6 (Fig. 3).

Therefore, it is possible that at low pH, such as in the stomach, the lysosome-alginate beads can maintain their morphology and lysosome contents. Moreover, at neutral pH, such as in the intestine, the beads can release lysosomes, allowing the treatment of infection with pathogens. This study presents the possibility of using a lysosome-alginate combination as an oral delivery system.

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