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Defensin carriers for better mucosal immunity in the digestive system

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ABSTRACT

The innate immunity utilizes a battery of broad-spectrum antibacterial cationic polypeptides named defensins. In humans, defensins are the first line of defense against pathogens, and their expression has been implicated in several diseases. In addition to exerting direct antimicrobial effects, defensins facilitate and amplify innate and adaptive immune responses. HD-5 is a polypeptide that plays a pivotal role in combating bacteria in the digestive system. Our results show that HD-5 can be entrapped within alginate carriers and strengthen their structure without changing their brittleness. In addition, carrier-entrapped HD-5 is released when incubated in buffer and/or stomach-simulating solution and still retains its activity after the release. This incubation also led to a decrease in carrier strength as well as an increase in their brittleness. Nevertheless the carriers did not disintegrate and remained intact throughout the diffusion process. The release of the defensin exhibited a bimodal behavior, suggesting that it was found both in a cross-linked and non-cross-linked form within the carrier. These results indicate that defensins encapsulated within alginate carriers could possibly be used for better mucosal immunity in the digestive system.

1. Introduction

The resident commensal microflora and microorganisms in the diet represent a continuing infectious challenge to the epithelial barrier of the gut. Although the nutrient-rich lumen of the small intestine would appear to provide a favorable environment for microbial cell growth, it contains few bacteria relative to the numbers found in the colon. α-Defensins are crucial for gut protection due to their antibacterial and immunomodulatory activities. α-Defensins are abundant constituents of mouse and human Paneth cell granules (Ouellette, 2004; Cunliffe, 2003). Secretion of α-defensins from Paneth cells constitutes a key source of antimicrobial peptide activity in the crypt lumen. In mammals, defensins are the predominant antimicrobial proteins, as evidenced by the large number of expressed genes, the various forms, and the ubiquitous occurrence in inflamed or infected tissues (Ganz, 2003). All defensins identified to date have the capability to kill and/or inactivate a spectrum of bacteria, fungi, or some enveloped viruses *in vitro* (Yang et al., 2002; Froy, 2005). In addition to exerting direct antimicrobial effects, defensins facilitate and amplify innate and adaptive immune responses, such as activation and degranulation of mast cells, interleukin and tumor necrosis factor production, and maturation of dendritic cells (Yang et al., 2002; Lehrer and Ganz, 2002; Lin et al., 2004).

Alginate beads have been investigated as drug carriers for the controlled delivery of low-molecular-weight compounds. The gelling strength and type of release in such beads are highly dependent on the type and concentration of the gel-inducing ions and hydrocolloid grades, gelling time, curing time, release-testing medium, the selected drug, the bead size, and formulation compositions (Shin et al., 1996). The best studied method of entrapment is sodium-alginate gelation by cross-linking via di- or multi-valent ions. Such gelation is easy to perform: drug substances or cells are mixed with sodium-alginate solution and added dropwise into a dilute calcium chloride bath. The strength and porosity of the beads can be somewhat controlled by the choice of alginate composition, i.e., the higher the L-guluronic acid content, the stronger the gel is (McDowell, 1960; Glicksman, 1969; Nussinovitch, 1997). If alginate with a higher proportion of D-mannuronic acid is chosen for production, a bead with a larger internal pore size is produced. As the concentration of alginate in the beads increases, so does their mechanical strength. If cells are included within the beads, an increase in cell mass has the reverse effect on bead strength (Nussinovitch, 1994, 2003; Nussinovitch et al., 1994).

Alginate gel beads can be reinforced by chitosan, a polycation, which forms a complex with them. This behavior leads to inhibition of the initial release rate of encapsulated drugs (Murata et al., 1993). Polyelectrolyte-coated alginate microspheres show

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promise as release systems to improve biocompatibility (Jayant et al., 2009). In addition, chitosan-coated alginate beads containing poly(N-isopropylacrylamide, PNIPAAM) can be used as a controlled pH/temperature-sensitive drug-delivery system with advanced encapsulation efficiency and delayed release rate (Shi et al., 2006). However, these beads have limitations, such as rapid erosion and a high release rate at neutral pH for dual-stimulus-responsive drug delivery. To overcome these limitations, the alginate gel matrix surface can be modified by ionic interactions with alginate carboxylate ions by macromolecules. Consequently, macromolecules could coat the alginate gel, with chitosan and poly(L-lysine) as typical surface modifications (Anal and Stevens, 2005; Ribeiro et al., 2005; Tapia et al., 2005). Although the concept of using polyanion–polycation interactions to produce or reinforce surface of carriers is not new, using a small polycation to reinforce both the surface and matrix as well as regulate its delivery in two stages, to the best of our knowledge has never been developed.

A disturbed antimicrobial defense seems to be a critical factor in the pathogenesis of Crohn's disease, an inflammatory disease of the intestinal tract (Wang et al., 2007). Human defensin 5 (HD-5) kills a variety of bacteria, including *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Vibrio cholerae*, as well as fungi, such as *Candida albicans* (Porter et al., 1997; Ghosh et al., 2002). A recent study has shown that in addition to HD-5 and HD-6, human neutrophil α-defensins, HNP-1–3, are also expressed in intestinal epithelial cells during active inflammatory bowel disease (Cunliffe et al., 2002). Enteric α-defensins were found to be crucial for survival in mice, as transgenic mice expressing HD-5 in Paneth cells of the small intestine were markedly resistant to oral challenge with virulent *S. typhimurium* (Salzman et al., 2003). Thus, increasing the concentration of human enteric α-defensin HD-5 may confer mucosal protection. Recently, an expression system has been established, in which high yields (milligrams) of HD-5 can be achieved. Analyses of the recombinant HD-5 yielded a protein with the same activity as the wild type HD-5 (Froy and Chapnik, unpublished data). We developed an alginate carrier that entraps HD-5 and could be used as a means to increase HD-5 concentrations in the small intestine and confer better mucosal immunity.

2. Materials and methods

2.1. Carrier preparation and treatments

Sodium-alginate powder with a molecular mass of 60–70 kDa containing 61% mannuronic acid and 39% guluronic acid (Sigma, Israel) was dissolved in doubly distilled water at room temperature (2% w/w) on a magnetic stirrer. The alginate solution was dropped into 2% $CaCl_2$ solution to obtain alginate beads by a spontaneous cross-linking reaction. The formed carriers (beads) (Fig. 1) were kept in the cross-linking solution for 24 h. Then, they were transferred to deionized water and rinsed three times with ~100 times the volume of the carriers. The deionized water was replaced 3 times (every 8 h) to remove excess non-reacting $CaCl_2$. The formed beads (3–4 mm diameter) were kept for additional 24 h in deionized water for swelling and then the surrounding fluid was discarded. The swollen beads were individually transferred to eppendorf test tubes containing 100 μl HD-5 dissolved in 10 mM PIPES buffer pH 7.4. Each bead reacted with the protein solution for 24 h at 4 °C. Release of HD-5 from the carrier was measured spectrophotometrically (see below) after incubation in 10 mM PIPES pH 7.4 for 2 h, in simulated gastric fluid for 2 h, or in simulated intestinal fluid for 6 h at 37 °C with gentle shaking (see below). Carriers containing HD-5 were dissolved in 2% sodium citrate (Sigma, Israel).

Fig. 1. Alginate carriers.

2.2. Expression and purification of HD-5

HD-5 gene was cloned into pET23b downstream of the gene encoding Bj-xtrIT, since Bj-xtrIT is known to be imported into *E. coli* inclusion bodies, as was previously reported (Froy et al., 1999). pET23b harboring Bj-xtrIT–HD-5 transformed into *E. coli* BL21 was grown at 37 °C for 18 h. The culture was then diluted 100-fold in LB and grown to OD_{600} = 0.2–0.4. 0.4 mM IPTG (isopropyl-thio-β-D-galactoside) was then added and incubation continued for another 5 h. Cells were harvested by centrifugation (10 min at 5000 × *g* at 4 °C) and resuspended in ddH_2O. Cell suspension was frozen and thawed to disrupt cell walls and total lysis was achieved by sonication. Cell lysates were centrifuged for 15 min at 20,000 × *g* and the pellet of inclusion bodies was washed and precipitated twice in washing solution (25% (w/v) sucrose, 5 mM EDTA, 1× PBS). HD-5 was highly expressed and found in the inclusion body fraction. The inclusion bodies containing the expressed defensins were dissolved in formic acid and CNBr (Sigma, Israel) was used to cleave HD-5 from Bj-xtrIT. The formic acid was evaporated and the protein resuspended in 10 mM pipes pH 7.4 in which HD-5 renaturation occurred. The solution was filtered and run on an RPC reverse phase HPLC column. Using quantitative amino acid analysis, the extinction coefficient for HD-5 was determined and found to be 16 μg/ml. Thereafter quantification was determined spectrophotometrically using the formula: $[\mu g/ml] = (OD_{228.5} - OD_{234.5}) \times 16$ (Froy et al., 1999).

2.3. Antimicrobial assays

Recombinant wild type HD-5 was tested for microbicidal activity against DH5α *E. coli*, as was previously described (Satchell et al., 2003). Briefly, bacteria growing exponentially at 37 °C in trypticase soy broth (TSB) were centrifuged at 1700 × *g* for 10 min, washed in 10 mM PIPES (pH 7.4) and resuspended in 10 mM PIPES (pH 7.4). The microorganisms were incubated at 37 °C with HD-5 in a total volume of 1 ml at a concentration of $\sim 1.25 \times 10^5$ ml^{-1} for 1 h in a shaking incubator. 25 μl samples of peptide-exposed bacteria were plated on TBS agar plates. The surviving bacteria were counted as colony-forming units after incubation at 37 °C for 12 h.

2.4. Mechanical properties of the carrier

Carriers were compressed between lubricated parallel plates to failure at a constant deformation rate of 10 mm/min with an Instron Universal Testing Machine (UTM) model 5544, connected to an IBM compatible personal computer using a card. Data acquisition and conversion of the Instron's continuous voltage vs. time output into digitized force vs. time relationship was performed by software (Merlin) from Instron Corporation (Canton, USA). Finally, the force vs. time data was converted to pseudo-stress vs. engineering strain relationships using the following equations: $\sigma = F/A_0$ and $\varepsilon_E = \Delta D/D_0$ in which σ is the engineering stress (Pa), ε_E is the dimensionless engineering strain, F is the force at a given time (N),

A_0 is the initial cross-sectional area of the carrier (m^2), ΔD is the absolute deformation caused by the compression (m) test, and D_0 is the diameter of the carrier at time zero (m).

2.5. Simulated gastric and intestinal fluids

Simulated gastric fluid was prepared by dissolving 10 mg of pepsin from porcine stomach mucosa (Sigma, Israel) in 112 ml of doubly distilled water, followed by the addition of 350 mg of mucin from porcine stomach (Sigma, Israel). Then, 3.5 ml of 3 N NaCl solution and 2 ml of 1.2 N KCl were added to the simulated gastric fluid. The pH was adjusted and kept at pH 1.2 by adding an appropriate volume of 1 N HCl (Hack and Selanka, 1996). Simulated intestinal fluid was obtained by titrating 120 ml of the simulated gastric fluid with sodium bicarbonate (Frutarom Ltd., Israel). The pH value was adjusted and kept at pH 6.8. Trypsin (10 mg) from porcine pancreas (Sigma, Israel), 350 mg of pancreatin from porcine pancreas (Sigma, Israel), and 350 mg of dried bovine bile (Sigma, Israel) were added to obtain the simulated fluid (Gal and Nussinovitch, 2007). Different incubation times, 2 h in simulated gastric fluid and 6 h in simulated intestinal fluid, mimic the duration of food in the stomach and small intestine, respectively, as has been established (Hack and Selanka, 1996; Washington et al., 2003).

2.6. Statistical analyses

Statistical analyses were conducted using JMP software (SAS Institute 2007, Cary, NC), including ANOVA and Tukey–Kramer Honestly Significant Difference test for comparisons of means, $p \leq 0.05$ was considered significant.

3. Results

3.1. Entrapment of HD-5 within alginate carriers

HD-5 was tested for its ability to be entrapped within alginate carriers. To verify that incubation of recombinant HD-5 led to its diffusion into and reaction with the carrier, carriers were weighed and their diameter was measured before and after incubation using an analytical scale and a digital caliper (Precisa Instruments, Switzerland). As a result of shrinking due to the cross-linking of the alginate by the HD-5 reaction, the weight of the carrier was decreased by 2.42% (Table 1). In addition, the mechanical strength of the carrier evaluated before and after the incubation by compression tests, was increased from 0.135 to 0.151 kPa (Fig. 2, Table 1). The strain at failure was 0.56 and remained constant. To study the amount of HD-5 in each carrier, the carriers containing HD-5 were dissolved and run on an SDS-PAGE. The amount of recombinant HD-5 in the carrier was evaluated to be 2 μg/carrier (data not shown).

3.2. HD-5 release from carriers incubated in buffer

After having established that HD-5 can be entrapped within alginate carriers, release of HD-5 from the carrier and its biological

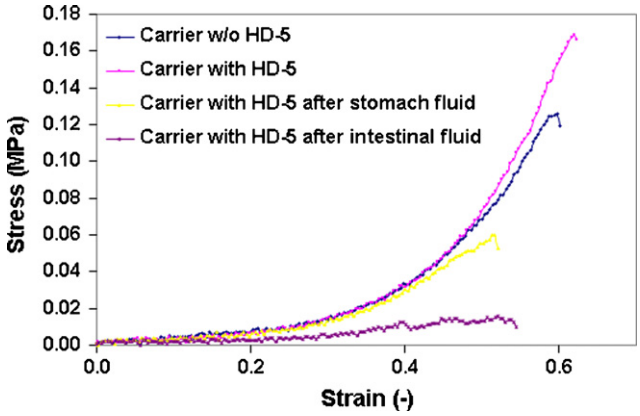

Fig. 2. Stress–strain relationships for blank carriers, carriers cross-linked with HD-5, and cross-linked carriers after immersion and release of HD-5 in stomach- and small intestine-simulating solutions.

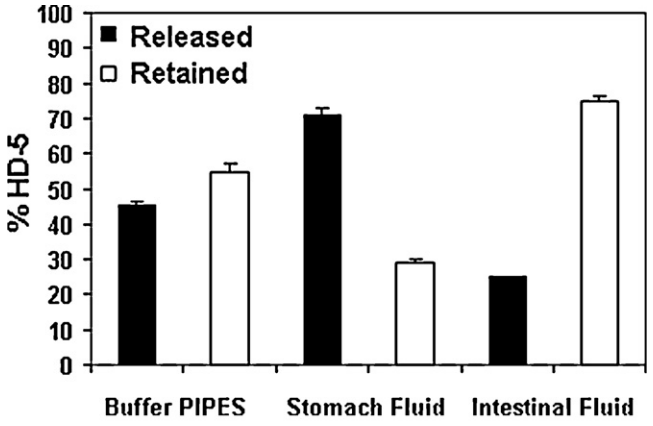

Fig. 3. Retained and released HD-5 levels after carrier incubation in PIPES buffer and stomach- and small intestine-simulating solutions.

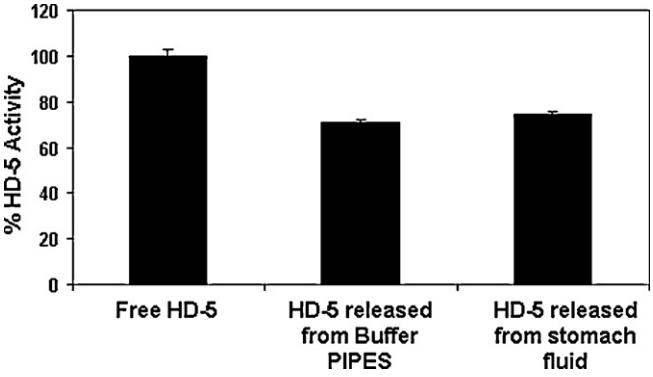

Fig. 4. Biological activity of released HD-5 after carrier incubation in PIPES buffer and stomach-simulating solution.

Table 1
Mechanical properties of HD-5-containing carriers.

	Weight (mg) (n)	Δ % weight[a]	Stress at failure (kPa)[b]	Engineering strain at failure[b]
Carrier w/o HD-5 in buffer PIPES[b]	68.49 ± 0.38 (50)	0	0.135 ± 0.011a	0.56 ± 0.03a
Carrier with HD-5 in buffer PIPES[b]	67.21 ± 0.47* (40)	−2.42 ± 0.11	0.151 ± 0.010b	0.56 ± 0.05a
Carrier with HD-5 in stomach fluid[b]	67.04 ± 0.55* (16)	−2.1 ± 0.21	0.058 ± 0.004c	0.52 ± 0.03ab
Carrier with HD-5 in intestinal fluid[b]	69.16 ± 1.1* (8)	0.41 ± 0.04	0.019 ± 0.008d	0.46 ± 0.08b

[a] Positive number—gain of weight, negative number—reduction in weight due to shrinking after cross-linking, n—number of repetitions.
[b] Results are expressed as mean ± standard error.
* Indicates significant weight changes ($p < 0.05$) compared with the carrier not containing HD-5. Different alphabets (a–d) indicate statistically significant differences at $p < 0.05$.

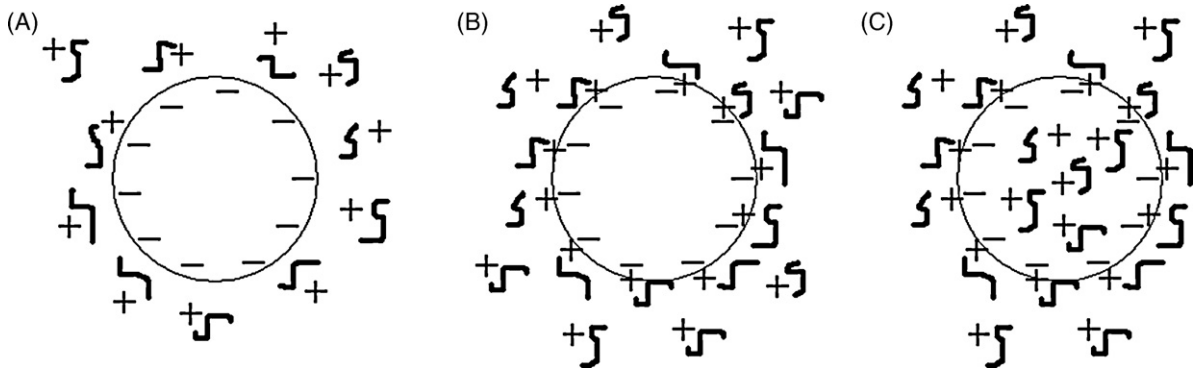

Fig. 5. Schematic model of HD-5 encapsulation within alginate carriers. (A) After immersion of the carrier in HD-5 solution and before the cross-linking; (B) after cross-linking at the surface of the carrier; (C) after further diffusion of the HD-5 from the surface into the carrier, reaching a situation where cross-linked HD-5 and non-cross-linked HD-5 are present within the carrier.

activity were measured. Released HD-5 after incubation of HD-5-containing carriers in buffer was measured spectrophotometrically. These experiments showed that 45% of the HD-5 was released whereas 55% was still bound to the carrier (Fig. 3). This indicated that the first mode of release due to HD-5 molecules found in the carriers but not cross-linked to the matrix. To test whether the released HD-5 still retains its biological activity, the released HD-5 was tested against *E. coli*. The activity of the released HD-5 was 75% of its original activity (Fig. 4).

3.3. HD-5 release from carriers incubated in stomach- and small intestine-simulating solutions

The ability of the carriers containing HD-5 to withstand enzyme-containing stomach and small intestine environments was tested next. HD-5-containing carriers were incubated in stomach- and small intestine-simulating solutions (Gal and Nussinovitch, 2007) for 2 h and 6 h, respectively. The weight of the carriers incubated in stomach-simulating solution decreased by ~2%, whereas that of the carriers incubated in small intestine-simulating solution increased by ~0.4% (Table 1). The compression test demonstrated that the carriers immersed in the stomach fluid decreased their average strength from 1.51 to 0.058 kPa (a reduction of ~60%), whereas a small non-significant decrease was observed in their strain at failure. Further decrease to 0.019 was detected for carriers that were incubated in intestinal fluid. The strain at failure was less influenced by the immersion in the different fluids and decreased to 0.46.

The solutions were then measured spectrophotometrically alongside the dissolved carriers. The results showed that ~71% and ~25% were released from the carriers in the simulated gastric and intestinal fluids, respectively. In addition, the solutions with the released HD-5 were tested for their biological activity against *E. coli*. Measuring the activity of HD-5 tested whether the released HD-5 still retained its biological activity after incubation in stomach and small intestine solutions. The biological activity of the released HD-5 entrapped in carriers incubated in stomach-simulating solution was ~71% (Fig. 4). However, the activity of the HD-5 released from carriers incubated in small intestine-simulated solutions could not be detected. These experiments showed the stability of HD-5 along the digestive system and the kinetics of release.

4. Discussion

The experiments with simulated gastric and intestinal fluids demonstrated that HD-5 stays intact after release from the carrier after incubation with enzyme-containing stomach-simulating solution. Differences in the release levels among PIPES buffer, simulated stomach fluid, and simulated intestinal fluid could stem from the effect of the corresponding solution pH on the interactions between HD-5 and the carrier matrix. Measuring the activity of HD-5 after incubation with buffer and simulated gastric fluid revealed that released HD-5 retained its biological activity until it is supposed to reach the small intestine, its site of action. No activity could be detected in the simulated intestinal fluid, presumably due to the longer period of incubation that led to HD-5 degradation. In general, these experiments show that HD-5 exhibits a bimodal release behavior, i.e., it can be both chemically cross-linked within alginate carriers and entrapped within them. Furthermore, the shrinkage of the carriers demonstrated the ability of recombinant HD-5 to react as a polycation with the alginate polyanion carrier and cause both a decrease in weight, as a result of syneresis, and an increase in its stress at failure. Thus, it is hypothesized that the mechanism in which the HD-5 reacts and diffuses into and out of the alginate swollen bead, exhibits a bimodal behavior and occurs at two stages. At the first stage, the small polycation (~3 kDa) interacts with the negatively charged acid groups of the carrier. After the reaction on the surface of the bead, polycation molecules enter the solidified bead by diffusion and react with inner negatively charged groups (Fig. 5). The diffusion of small polycation into the beads is not surprising, since it was previously reported that for both alginate beads and alginate liquid-core capsules, substances (proteins) with molecular weights of up to 154 kDa can diffuse in or out of the carrier and that their rate of diffusion is dependent on their molecular weight (Nussinovitch et al., 1996). Diffusion occurs until most, if not all, of the polyanion is cross-linked by the polycation. In addition, upon further diffusion, HD-5 accumulates within the free volume of the bead. This hypothesized model explains why after incubation of carriers with buffer, the entrapped HD-5 can be released spontaneously from the carrier, while there is still reacted HD-5 that is more strongly bound to the polymer network. These results demonstrate that alginate carriers containing HD-5 can be used to deliver HD-5 into the digestive system with the goal of obtaining better mucosal immunity.

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