Carbopol-Lectin Conjugate Coated Liposomes for Oral Peptide Delivery

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Within the current study, a delivery system based on a novel polymer-lectin conjugate (carbopol-lectin) was evaluated for the oral delivery of therapeutic peptides and proteins. It was demonstrated that covalent attachment of lectin to carbopol does neither decrease nor abolish the specific binding properties of lectin. Bioadhesion studies revealed that liposomes coated with carbopol lectin are more bioadhesive than liposomes coated with unmodified carbopol. Finally, the *in vivo* data suggest that carbopol-lectin conjugate coated liposomes are effective oral peptide delivery systems which are capable of increasing the pharmacological effect of orally administered calcitonin.

Key words carbopol-lectin; oral peptide delivery; polymer-coated liposome; oral protein delivery; oral calcitonin delivery

Although the oral administration route can be regarded as the most convenient one, most therapeutic peptides and proteins are available only as injections. This is due to the low bioavailability of orally administered peptide drugs, which is caused by a number of barriers, including the enzymatic barrier[1] and the permeation barrier.[2] As oral peptide delivery is highly demanded by patients, various approaches have been developed in recent years to overcome these barriers and to achieve sufficient bioavailability after oral administration. One promising approach is the coating of liposomes with bioadhesive polymers such as chitosan or carbopol (CP). It has been demonstrated that such delivery systems exhibit increased bioadhesive properties and are capable of improving and prolonging the pharmacological effect of incorporated peptides, such as calcitonin or insulin.[3] Another approach is based on the specific bioadhesive properties of lectins. Wheat germ agglutinin (WGA) from *Triticum vulgaris* is a non-toxic glycoprotein which can specifically bind to *N*-acetyl-D-glucosamine.[4] This sugar is present on intestinal cells as well as in the intestinal mucus.[5] Recently, it was demonstrated that lectin-conjugated polylactic-co-glycolic acid (PLGA) nanoparticles display increased intestinal bioadhesion and furthermore increase the oral bioavailability of peptide drugs.[6,7] It was the aim of the study to (1) synthesize a novel polymer-lectin conjugate by covalently attaching WGA to carbopol, (2) investigate if the covalent attachment of WGA does decrease or abolish the binding activity of WGA to *N*-acetyl-D-glucosamine, (3) prepare CP-lectin coated liposomes, and (4) evaluate these coated liposomes concerning intestinal bioadhesion and effect on the oral absorption of incorporated calcitonin.

Results and Discussion

The novel CP-lectin conjugate was synthesized by covalently attaching the amino groups of WGA to the carbodiimide-activated carboxylic groups of carbopol (Fig. 1). Using a spectrophotometric method, the amount of WGA in the purified polymer-lectin conjugate was determined to be 11.79± 0.8% (mg lectin/100 mg of CP-lectin). To investigate, whether the covalently bound WGA was still capable of binding to *N*-acetyl-D-glucosamine, a haemagglutination test was performed. *N*-acetyl-D-glucosamine is, among others, present on the surface of erythrocytes. Therefore, erythrocytes agglutinate in the presence of WGA. Results of this study are shown in Fig. 2. Agglutination was clearly observed in the presence of unbound WGA as well as in the presence of CP-lectin. Contrary, no agglutination was observed in presence of unmodified CP. These results reveal that the novel CP-lectin conjugate is capable of binding to its substrate. Moreover, the comparison of the agglutination behaviour of various concentrations of unbound WGA and CP-lectin supports the calculations concerning the amount of WGA in the CP-lectin conjugate gained in the spectrophotometric studies. Next, we investigated if positively charged liposomes can be coated with the negatively charged CP-lectin. As shown in Table 1, the initial positive zeta-potential of the uncoated liposomes switched to a negative value after coating with CP and CP-lectin, respectively. This indicates the successful coating of the multilamellar vesicles (MLV). For bioadhesion studies, the fluorescence marker coumarin was incorporated into liposomes, which where subsequently coated with CP and CP-lectin. Four hours after intragastric administration of the formulations to rats, the small intestines were isolated and divided into three parts. Results of confocal laser scanning microscopy studies suggest that the observed fluorescence on the mucosal sides of rats which received CP-lectin MLV was more intense than that of the control group receiving CP-MLV (Fig. 3). In particular, fluorescence was observed in the duodenum of the CP-lectin MLV group, whereas no fluorescence was observed in the CP-MLV group, indicating stronger bioadhesive properties of CP-lectin MLV. Finally, the blood calcium level was determined after oral administration of CP- and CP-lectin MLV containing calcitonin. As shown in Fig. 4, the blood calcium level decreased after administration of both formulations, but was significantly lower after 12 and 24 h in the case of CP-lectin MLV. Comparing the area above the curve of the CP MLV and the CP-lectin MLV groups revealed that the CP-lectin coated MLV were more than 6-times effective than the CP-MLV. Lectins are capable of specifically binding to *N*-acetyl-D-glucosamine and sialic acid moieties on both M-

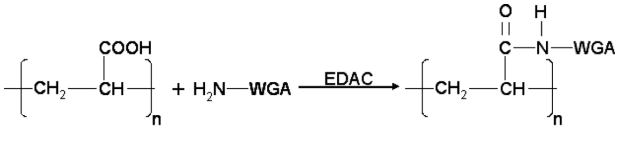

Fig. 1. Schematic Presentation of WGA Conjugation to Carbopol

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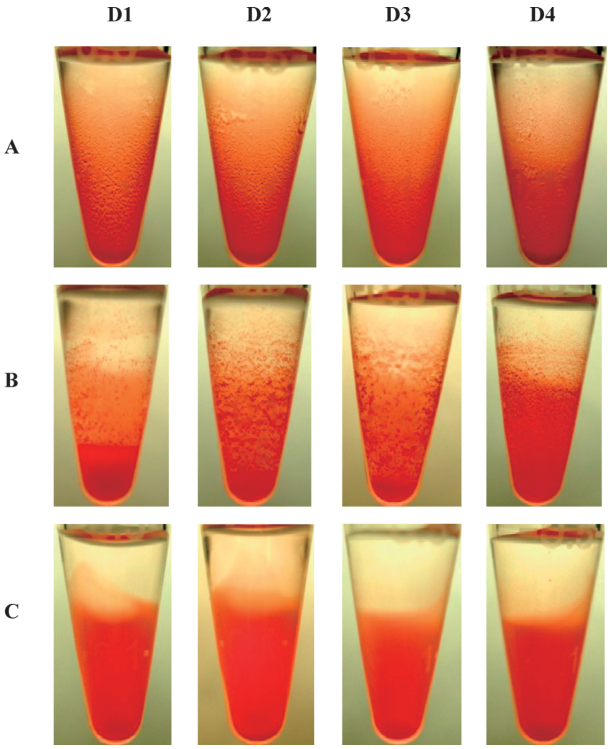

Fig. 2. Haemagglutination in the Presence of Different Concentrations (D1—D4) of (A) Lectin (from 0.1 to 0.00313 mg/ml of Free WGA), (B) CP-Lectin and (C) Carbopol (from 5 to 0.156 mg/ml of Polymer) after 2 h Incubation at Room Temperature

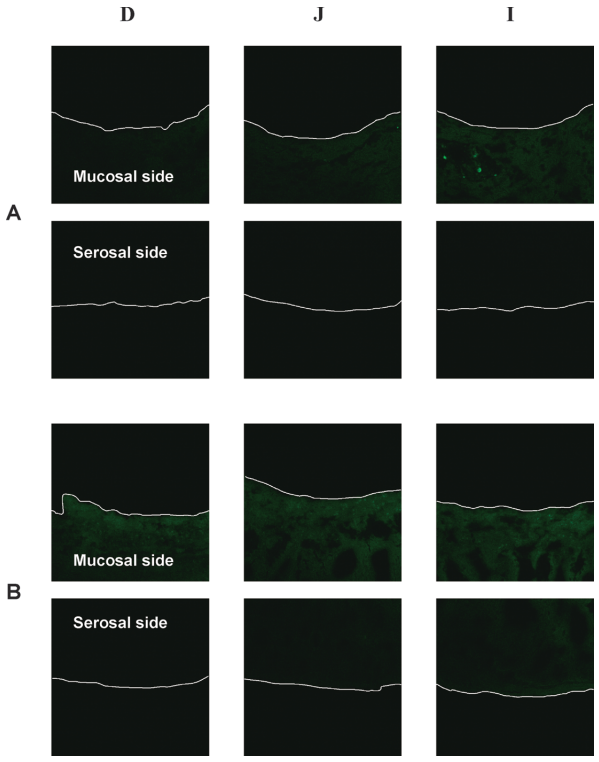

Fig. 3. Confocal Laser Scanning Micrographs of Different Segments of Rat Intestinal Mucosa (D=Duodenum, J=Jejunum, I=Ileum) 4 h after Peroral Administration of Coumarin Loaded Liposomes; (A) CP-MLV and (B) CP-Lectin MLV

Table 1. Size and Zeta-Potential of Uncoated, CP-Coated and CP-Lectin Coated MLV

Formulation	Size (μm)	Zeta potential (mV)
Uncoated	5.12	+37.8±6.1
CP-coated	5.68	−70.3±10.8
CP-lectin coated	5.41	−62.9±4.3

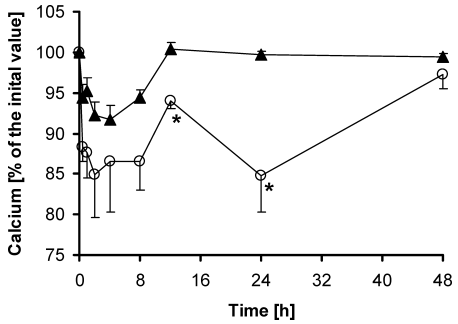

Fig. 4. Blood Calcium Profiles after Intragastric Administration of Calcitonin Loaded CP-MLV (▲) and CP-Lectin MLV (○), Administered Dose: 40 μg Calcitonin per Rat

$*p<0.05$; each point represents the mean±S.E.M. of six experiments.

cells and regular enterocytes of the intestinal membrane.[6] This specific bioadhesion on the epithelial cells, rather than general entrapment onto the mucous gel layer, could extend the residence time of the delivery system and achieve higher local drug concentration at the absorption membrane. Furthermore, lectin-mediated cytoadhesion can trigger the active transport of large molecules or the lymphatic uptake of particulate system.[8] In conclusion, the novel carbopol-lectin conjugate appears to be an effective and promising coating material for improving oral delivery of peptide and protein drugs using particulate systems.

Experimental

Materials Carbopol 971P-NF (CP, BF Goodrich), wheat germ agglutinin from *Triticum vulgaris* (WGA, Sigma), *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDAC, Sigma), L-α-distearolyphosphatidylcholine (DSPC, Nippon Oil and Fats Co.), cholesterol (Chol, Sigma), stearylamine (SA, Tokyo Kasei), and Coumarin-6 (Sigma) were used as received. Calcitonin (elcatonin) was kindly supplied by Asahi Chemical Co., Japan. All used reagents were of analytical grade.

Synthesis and Characterization of CP-Lectin WGA was covalently attached to carbopol using the carbodiimide method. Typically, 50 mg of carbopol were dissolved at room temperature in 15 ml of purified water. The pH was adjusted to 6.0 by the drop wise addition of NaOH. The carboxylic groups of carbopol were activated by the addition of 300 mg of EDAC dissolved in 2 ml of purified water. The pH was adjusted to 6.0 and the mixture was incubated under stirring at room temperature for 3 h. Afterwards, 10 mg of WGA were dissolved in 10 ml of water and added to the activated carbopol solution. The mixture was incubated at room temperature under stirring for 24 h. To remove unbound WGA and EDAC, the reaction mixture was filled into dialysis tubings (MWCO=50 kDa) and dialysed 6 times for 12 h against water. Finally the product was lyophilised. The amount of WGA in the conjugate was determined by measuring the absorbance (272 nm) of various concentrations of CP-lectin. After substraction of the absorbance of CP, the values were calculated using a calibration curve performed with various concentrations of WGA.

Haemagglutination Test A 2% dilution of freshly collected rat blood in phosphate buffer saline (PBS) buffer pH 7.4 was prepared. To 250 μl of the blood dilution, 250 μl of different concentrations of CP and CP-lectin in PBS pH 7.4 (from 5 to 0.156 mg/ml of polymer) or lectin (from 0.1 to 0.00313 mg/ml of WGA in PBS pH 7.4) were added and incubated at room temperature for 2 h. Agglutination was observed optically.

Preparation and Characterisation of Liposomes and Coated Liposomes Preparation of liposomes coated with poly(acrylates) has been described previously.[9] Cationic multilamellar liposomes (MLV) consisting of

DSPC, SA and Chol (molar ratio: 8 : 0.2 : 1) were prepared using the thin film method. For bioadhesion studies, the fluorescent marker coumarin was added to the organic solution. Hydration of the thin film was performed with *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer pH 7.4 by repeated gentle heating and vortexing. For *in vivo* absorption studies, calcitonin was added to the hydration medium. Finally, the liposome suspension was incubated for 30 min at 10 °C. Polymer coating was performed by mixing an aliquot of the described liposomal suspensions with a 0.6% polymer solution (CP and CP-lectin, respectively) in HEPES pH 7.4 to obtain CP-MLV and CP-lectin MLV. The size of MLV was determined using a LDSA 2400A particle size analyzer (Tohnichi Computer Co., Ltd., Japan) and the zeta potential of all liposomes was measured using a Zetasizer from Malvern.

Bioadhesion Studies Liposomes containing coumarin (prepared as described above) were hydrated with HEPES pH 7.4 to give a final coumarin concentration of 0.1 mg/ml in the liposomal suspension after polymer coating. CP-MLV and CP-lectin MLV suspensions (500 μl, respectively) were administered intragastrically to 10 week old rats which had been fasted for 48 h prior to administration. Four hours after administration, the intestines were excised and divided into three segments referred to as "duodenum," "jejunum" and "ileum." Each segment was washed with 10 ml of 0.9% saline solution. Samples were frozen and sliced into sections of 10 μm thickness by using a Cryostat (LEICA). Samples were analysed using a confocal laser scanning microscope (LSM510, Carl Zeiss Jena) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

***In Vivo* Studies** All animal experiments performed within the current study were approved by the animal welfare commission of Gifu Pharmaceutical University. Calcitonin containing MLV were intragastrically administered to 10 week old male Wistar rats which were fasted 24 h prior to the experiment. Calcitonin concentration was 40 μg per rat. During the experiment, rats had free access to water. One milliliter of CP-MLV or CP-lectin MLV was administered to each rat of each group (n=6 rats per group). Blood samples (200 μl) were withdrawn from the jugular vein after predetermined time-points (0.5, 1, 2, 4, 8, 12, 24, 48 h). Blood calcium levels were determined by using a commercially available calcium kit (Calcium C-Test, WAKO, Wako Pure Chemicals, Japan).

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