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Research paper

In vitro and in vivo evaluation of WGA–carbopol modified liposomes as carriers for oral peptide delivery

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ABSTRACT

Surface modification of liposomal nanocarriers with a novel polymer–lectin conjugate was proposed for enhancing the systemic uptake of encapsulated peptide and protein therapeutics after oral administration. Wheat germ agglutinin (WGA) was covalently attached to carbopol (CP) using the carbodiimide method. The prepared WGA–CP conjugate retained the biological cell binding activity of WGA without any evidence of cytotoxicity to Caco-2 monolayers. Cationic liposomes in the size range of 100 nm were prepared by the lipid film hydration method followed by probe sonication and surface modification with negatively charged WGA–CP. The uptake of WGA–CP liposomes by Caco-2 cells was significantly higher than that of non-modified or CP liposomes. The uptake was dependent on the surface concentration of WGA, temperature, and incubation period and was significantly inhibited in the presence of chlorpromazine and 10-fold excess of free WGA. These results suggest the involvement of active transport mechanism for the cellular uptake of the modified liposomes, mediated mainly by binding of WGA to its specific cell membrane receptors. Dual channel confocal microscopy confirmed the simultaneous association and internalization of the polymer conjugate and the liposomal carrier by Caco-2 cells and intestinal membrane of rats. In addition, the pharmacological efficacy of calcitonin, a model peptide drug, was enhanced by more than 20- and 3-fold following peroral administration of calcitonin-loaded WGA–CP liposomes when compared to non-modified and CP liposomes, respectively.

1. Introduction

Despite the steadily increasing importance of biopharmaceuticals, including peptide and protein therapeutics, most of these drugs are available only as injections. The peroral delivery of these agents is hampered by their poor membrane permeability and enzymatic degradation during gastrointestinal (GI) passage. Entrapment of protein therapeutics into micro-/nanoparticles and liposomes has been frequently reported to improve their systemic absorption after peroral administration [1–5]. Particulate carriers can protect the entrapped protein against the harsh environment of the GI tract and control the release properties of the entrapped drug [6]. The attachment of the carrier system to the intestinal mucosa can also facilitate the epithelial transport of the drug via the paracellular route or by direct translocation of the intact particles. It is generally agreed that particles below 1 μm, and preferably below 200 nm, can be absorbed through epithelial membranes by adsorptive or clathrin-mediated endocytosis [7–9]. This uptake behavior of colloidal nanocarriers by intestinal epithelium is quite sufficient for the induction of local immune response for vaccination purposes. However, it is still too low to achieve efficacious therapeutic plasma levels of orally administered peptide and protein therapeutics [10,11]. Therefore, the membrane association and the uptake properties of submicron-sized carriers need to be further enhanced to achieve the desired therapeutic level of the entrapped drug.

Lectins are glycoproteins available from different plant species that can recognize and bind to their specific carbohydrate moieties on the mucosal membranes. Their receptor binding is as specific as enzyme–substrate or antigen–antibody interaction [12]. Carbohydrate recognition by lectins forms the basis for their use in drug targeting. The targeting approach utilizes exogenous lectins as targeting moieties that facilitate the binding and uptake of the delivery system onto the surface of cells. By virtue of their direct cell adhesion, lectins can trigger the active transport of large molecules or nanocarriers by active transport mechanisms [13]. For this purpose, different types of plant lectins are being actively investigated to target drugs to the intestine, colon, or various tumors that overexpress lectin receptors [12,14]. Wheat germ agglutinin (WGA, 36 kDa) is a non-toxic and non-immunogenic lectin derivative isolated from *Triticum vulgaris*. It can specifically bind to N-acetyl-D-glucosamine and sialic acid residues present in the glycocalyx of the intestinal

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enterocytes as well as in the intestinal mucus [15]. It was demonstrated that WGA does not only bind to its cell membrane receptors, but also could be taken inside the cells.

Recently, we have shown that multilamellar vesicle (MLV) liposomes (5–6 μm) surface modified with a novel wheat germ agglutinin–carbopol (WGA–CP) conjugate could be effectively retained on the intestinal membrane of rats and enhance the oral absorption of the entrapped peptide after peroral administration [4]. In the current study, the feasibility of surface modifying small unilamellar vesicle (SUV) liposomes of 100-nm diameter with WGA–polymer conjugate was explored. The epithelial association and uptake mechanism of the lectin modified liposomes were investigated using in vitro Caco-2 cells by quantitative fluorescence analysis and confocal laser scanning microscopy (CLSM). The ability of the modified liposomes to transverse the intestinal barrier after peroral administration to rats was also investigated by CLSM observation of the small intestine and by assessment of the pharmacological activity of the entrapped model peptide, calcitonin.

2. Materials and methods

2.1. Synthesis and evaluation of wheat germ agglutinin–carbopol (WGA–CP) conjugate

WGA was covalently attached to CP using the carbodiimide method [4]. Typically, 50 mg of CP (1250 kDa, BF Goodrich, USA) was dissolved at room temperature in 15 mL of MiliQ water, and the pH was adjusted to 6.0 by the drop-wise addition of NaOH. The carboxylic groups of CP were activated by the addition of 300 mg of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC, Sigma, USA). 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 (36 kDa, Sigma, USA) was added, and the mixture was incubated at room temperature under stirring for 12 h. To remove unbound WGA and EDAC, the reaction mixture was filled into dialysis tubing (MWCO = 100 kDa) and dialyzed six times for 3–4 days against distilled water. Finally, the product was lyophilized after freezing at −120 °C. For the preparation of fluorescently labelled WGA–CP conjugate, FITC–WGA (Sigma, USA) was utilized instead of WGA using the same procedures.

The absorption spectra of WGA, CP, and WGA–CP conjugate were evaluated using UV spectrophotometry (UV-1700, Shimadzu, Japan). The amount of WGA in the conjugate was determined by measuring the absorbance (275 nm) of various concentrations of WGA–CP solutions. After subtraction of the absorbance of CP, the values were calculated using a calibration curve for free WGA. The biological activity of WGA chemically conjugated to CP was evaluated by the haemagglutination test as previously described [4].

2.2. Cytotoxicity (MTS assay)

The potential toxic effects of CP and WGA–CP on Caco-2 cells were evaluated in vitro using the standard MTS assay. Caco-2 cell lines (#46–52) were obtained from the European Collection of Cell Cultures (ECACC, UK). The cells were cultured in 0.2-μm vented tissue culture flasks (BD Biosciences, USA) under standard conditions (5% CO_2 and 37 °C) using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 1% non-essential amino acids, and 1% penicillin–streptomycin solution. The cells were passaged using 0.25% Trypsin solution containing 0.2% EDTA. All the reagents utilized in cell studies were obtained from Invitrogen, USA.

Trypsinized Caco-2 cells were seeded onto polystyrene 96-well plates (BD Biosciences, USA) at a density of 3.15×10^4 cells/cm^2 and cultured for 7 days. The cells were washed three times with HBSS and incubated with 100 μl of each concentration of the polymer solutions for 2 h. After washing, the cells were further incubated for 2 h with 100 μl of DMEM and 20 μl of MTS reagent (Promega, USA). The absorbance of the produced color was measured with a microplate reader (MTB 120, Corona Electric, Japan) at a wavelength of 490 nm. Background absorbance in cell-free wells was measured and subtracted from the measurement absorbance values. Solutions of PBS and 0.1% sodium dodecyl sulfate (SDS) served as negative and positive controls, respectively. The percentage of cell viability was calculated using the following relation: $ABS_{Sample}/ABS_{PBS} \times 100$.

2.3. Preparation and evaluation of WGA–CP modified liposomes

The cationic multilamellar vesicles (MLV) were prepared using the thin film hydration method. Typically, a mixture of L-α-distearoylphosphatidylcholine (DSPC, Nippon Oil and Fats, Japan), stearylamine (SA, Tokyo Kasei, Japan), and Cholesterol (Chol, Sigma, USA) in a molar ratio of 8:0.2:1 was dissolved in 10 ml of chloroform. The solvent was evaporated to dryness, and the lipid film was further dried under vacuum overnight. The MLV liposomes were obtained by the hydration of the lipid film at 70 °C using phosphate buffer (pH 7.4) or FBS-free culture medium. The small unilamellar vesicles (SUV) were obtained by probe sonication for 5 min in ice water bath (Sonifier 250, Branson, USA) at output efficiency of 20%. Fluorescence labelling of the liposomal carrier was performed using Coumarin-6 (C6, Sigma, USA) or 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, LAMBDA, Austria). The hydrophobic markers were loaded during the preparation of the thin lipid film, and the liposomes were prepared as aforementioned.

For the in vivo absorption studies, calcitonin (Asahi Chemical, Japan) was loaded into the liposomes as a model of therapeutic peptides. Calcitonin loading was performed by dissolving the drug in the aqueous hydration medium prior to the preparation of the MLV liposomes. The entrapment efficiency of calcitonin in SUV liposomes was determined after ultracentrifugation (75,000 rpm, 45 min), and measurement of the free drug in the supernatant by Micro BCA protein assay (Pierce, USA).

Surface modification of positively charged liposomes with negatively charged CP or WGA–CP conjugate was mediated by electrostatic interaction. Modification of the surface of the prepared liposomes was accomplished by mixing an aliquot of the liposomal suspension with an equal volume of the polymer solution and vortexing. The particle size of SUV liposomes was determined by photon correlation spectroscopy (PCS), while zeta potential determinations were carried out by the method of laser Doppler electrophoresis (LDE) using Zetasizer nano (Malvern Instruments, UK).

2.4. Cellular association and uptake mechanism of WGA–CP liposomes

The cellular association of fluorescently labelled non-modified liposomes, CP liposomes, and WGA–CP liposomes was investigated in Caco-2 cell lines using quantitative fluorescence analysis and CLSM. Trypsinized Caco-2 cells were seeded onto polystyrene 24-well plates (BD Biosciences, USA) at a density of 3.15×10^4 cells/cm^2. The cells were cultured for 7 days, washed twice, and incubated with the different samples (500 μl/well) for 1, 2, or 4 h at 37 °C or 4 °C. The added liposomal dispersions had a lipid concentration of 4 mM, C6 concentration of 10 μg/ml, and surface polymer concentration of 1 mg/ml. After the predetermined time, the samples were removed, and the monolayers were washed twice with cold HBSS solution (4 °C, 500 μl/well). The cells were then lysed by adding 1 N NaOH (200 μl/well) and incubated at room temperature for 1 h in the dark. The cell lysates were diluted by adding MiliQ water (800 μl/well) and mixing. Five hundred

microliters of the cell lysate solution was mixed and shaken with 3 ml of chloroform/methanol mixture (1:1 v/v) in dark glass tubes. The amount of C6 in each well was determined (λ_{EX}: 490 nm, λ_{EM}: 520 nm, F-3010 fluorophotometer, Hitachi, Japan) and normalized to the protein content in the cell lysate (BCA protein assay, Pierce, USA). The effect of various metabolic inhibitors on the cellular uptake of WGA–CP liposomes was investigated. Cell monolayers were pre-incubated with chlorpromazine (10 μg/ml), filipin (1 μg/ml), or 10-fold excess free WGA for 30 min prior to the addition of the liposomal suspension.

The uptake of WGA–CP liposomes by single Caco-2 cells was visualized using dual channel CLSM. The liposomes were labelled red using DiI and surface modified with FITC–WGA–CP. Caco-2 cell suspensions (250 μl, 1×10^5 cells/ml) were incubated with 250 μl of the double-stained liposomes for 1 h at 37 °C. The cells were collected by centrifugation (1000 rpm, 5 min), and washed three times with 1 ml cold HBSS prior to mounting for microscopy. Confocal images were captured using a Zeiss LSM 510 confocal laser scanning system (Carl Zeiss, Germany) equipped with a laser operating at 488 nm and 543 nm for FITC and DiI excitation, respectively. The background offset of the green fluorescence detector was adjusted to eliminate any autofluorescence of the cells.

2.5. In vivo animal studies

Male Wistar rats (10–11 weeks, SLC, Japan) were used in all in vivo studies. All experiments were approved and monitored by the Institutional Animal Care and Use Committee of Gifu Pharmaceutical University, and in line with the Japanese legislation on animal studies. Before experiments, the rats were fasted for 24 h with free access to water.

2.5.1. Intestinal bioadhesion and penetration of WGA–CP liposomes

The mucosal association and penetration of C6-loaded liposomes were visualized by CLSM. The test samples were orally administered to the rats using intragastric tubes. The administered samples had a lipid concentration of 10 mM, C6 concentration of 25 μg/ml, and surface polymer concentration of 5 mg/ml. The rats were sacrificed at 2, 4 or 8 h after oral administration, and intestinal segments from duodenum, jejunum, and ileum were separated after a midline incision in the abdomen. The freshly excised tissues were cryofixed in Tissue-Tek® Compound by immersion into liquid nitrogen. The molded samples were sectioned (10 μm) using a cryomicrotome (Leica CM, Germany) and imaged at 488 nm for fluorescence excitation. For further elucidation of the role of WGA in the mucosal adhesion and penetration of the liposomal carrier, the liposomes were labelled with DiI and surface modified with FITC–WGA–CP. The rats were sacrificed 2 h after oral administration, and the intestinal segments were prepared for confocal microscopy as aforementioned. Dual channel confocal images were captured at 488 nm and 543 nm for FITC and DiI excitation, respectively.

2.5.2. Oral absorption of calcitonin

The oral absorption of calcitonin from the different liposomal formulations was evaluated by measuring the blood calcium level. Calcitonin solution or calcitonin-loaded liposomes was orally administered to each rat ($n = 4$–6) at a calcitonin dose of 20 μg/rat. Blood samples (200 μl) were withdrawn from the jugular vein after predetermined time points (0, 0.5, 1, 2, 4, 8, 12, and 24 h). Blood calcium levels were determined using a commercially available calcium kit (Calcium E, Wako, Japan). The areas above the blood calcium level curves (AAC) were calculated for the estimation of the relative pharmacological efficacy.

2.6. Statistical calculation

All the results were expressed as mean values ± SD. The Student's *t*-test was applied to study the significance of difference between two groups, while the one-way analysis of variance (ANOVA) followed by Tukey–Kramer test was used in the case of multiple comparison.

3. Results

3.1. Synthesis and characterization of WGA–CP polymer conjugate

The WGA–CP polymer conjugate was synthesized by covalently attaching the amino groups of WGA to the carbodiimide-activated carboxylic groups of carbopol [4]. The obtained conjugate was purified by repeated dialysis through a 100-kDa membrane to confirm the complete removal of EDAC and the unreacted WGA, and the final product was recovered through freeze-drying. The presence of WGA in the final product was qualitatively confirmed by UV spectrophotometry (data not shown). Quantitative estimation of the amount of WGA conjugated to CP was performed by calibrating free WGA at 275 nm. The amount of WGA in the purified polymer conjugate was determined to be 11.36 ± 0.83% (mg WGA/100 mg of WGA–CP conjugate). To investigate whether the covalently bound WGA is still capable of binding to its specific carbohydrate moieties on cell membranes, a haemagglutination test was performed. Test results confirmed the biological activity of the conjugated WGA as previously indicated [4]. Moreover, comparison of the lowest concentrations of unbound WGA and WGA–CP that induce a visually detected agglutination of blood cells supported the calculations concerning the amount of WGA in the modified polymer conjugate gained by UV analysis (data not shown).

3.2. Cytotoxicity (MTS assay)

Fig. 1 shows the percentage of Caco-2 cell viability after incubation for 2 h with 100 μl of each test sample. While SDS (1 mg/ml) significantly reduced the viability of Caco-2 cell cultures, both CP and WGA–CP conjugate did not result in any significant cytotoxicity even at the highest investigated concentration (4 mg/ml).

3.3. Preparation and evaluation of WGA–CP modified liposomes

SUV liposomes composed of DSPC, SA, and Chol (8:0.2:1 M ratio) were prepared for in vitro interaction with Caco-2 cells and in vivo animal studies. Tables 1 and 2 show the composition and physical properties of the prepared liposomal carriers. The non-modified liposomes had a particle size in the range of 95–130 nm

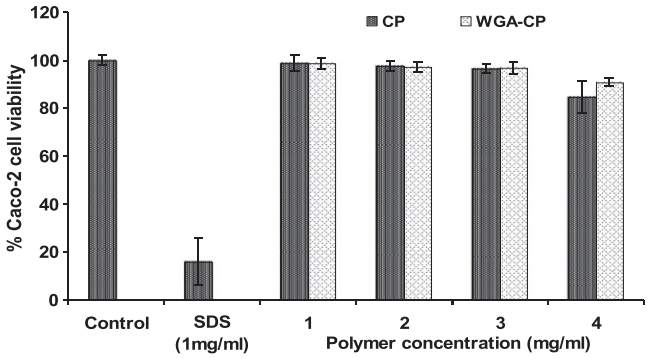

Fig. 1. Caco-2 cell viability after 2-h incubation with control buffer, SDS (1 mg/ml), and different concentrations of CP and WGA–CP (mean ± SD, $n = 8$).

Table 1
Composition and properties of SUV liposomes prepared for in vitro Caco-2 studies.

Formulation	Particle size (nm)	Zeta potential (mV)
Non-modified liposomes	94.99	12.8 ± 5.86
CP liposomes	194.6	−46.2 ± 4.38
CP/WGA–CP (1:1) liposomes	187.1	−43.6 ± 4.99
WGA–CP liposomes	191.8	−41.9 ± 4.80

Final lipid concentration; 4 mM, final coating polymer concentration; 1 mg/ml.

Table 2
Composition and properties of SUV liposomes prepared for in vivo animal studies.

Formulation	Particle size (nm)	Zeta potential (mV)
Non-modified liposomes	128.5	9.76 ± 3.65
CP liposomes	257.0	−54.6 ± 3.95
WGA–CP liposomes	201.3	−57.3 ± 3.85

Final lipid concentration; 10 mM, final coating polymer concentration: 5 mg/ml.

with positive surface potential. Coating of the liposomal surface with negatively charged CP or WGA–CP was confirmed by the inversion of the surface potential of the liposomal dispersions. The existence of the hydrophilic polymer coating layer around the liposomal particles resulted in an increase in the particle size of liposomes to about 200–250 nm. No difference was observed in the particle size and zeta potential of liposomes surface modified with the different coating polymers. In addition, the prepared liposomes were stable after incubation for 4 h in FBS-free culture medium at 37 °C without any noticeable change in particle size (data not shown).

3.4. Cellular association and uptake mechanism of WGA–CP liposomes

The entrapment efficiency of the hydrophobic marker C6 in the liposomes was approximately 100%, with no more than 2.0% released from the liposomes after 4-h incubation period at 37 °C in FBS-free medium. To characterize the cellular association and the uptake mechanism of C6 liposomes, the amount of fluorescent marker normalized to the protein content of each monolayer was determined. The effect of surface modification with CP and WGA–CP on the cellular association of the liposomes was investigated after an incubation period of 2 h at 37 °C (Fig. 2A). Surface modification with CP did not significantly influence the association behavior of the liposomes, indicating the absence of cell-specific interaction mechanism of CP with Caco-2 cells. On the other hand, the amount of liposomes associated with Caco-2 cell monolayers was found to increase by increasing the surface concentration of WGA. At the highest investigated surface concentration of WGA–CP (1 mg/ml), the cellular association was enhanced by approximately 2-fold when compared to the non-modified liposomes. The interfering effect of the released marker, obtained by ultracentrifugation of the liposomal dispersion after 4-h release period at 37 °C, was investigated. The contribution of the released dye to the total cellular association of the liposomes was negligible. This result indicates that C6 detected in the monolayers occurs as a result of cellular association of the liposomes rather than by marker release.

The effect of low temperature on the Caco-2 association of the different liposomal carriers was evaluated by incubation at 4 °C for 2 h (Fig. 2B). At 4 °C, significant reduction in Caco-2 cell association was observed in the case of WGA–CP liposomes, while the cellular association of the non-modified and CP liposomes was not significantly influenced. These results could indicate that the uptake of WGA–CP liposomes occurs via energy-dependent process. Despite the absence of significant difference, the cellular

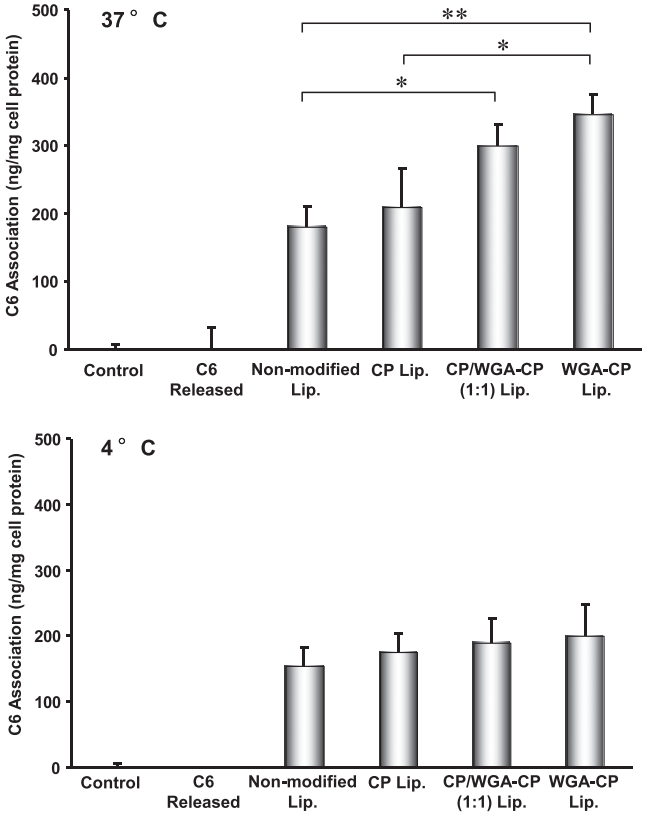

Fig. 2. Effect of surface modification with WGA–CP conjugate on liposomal association with Caco-2 cell monolayers after 2-h incubation at 37 °C or 4 °C (mean ± SD, $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).

association at 4 °C was also observed to increase by surface modification with WGA–CP.

The amount of liposomes associated with Caco-2 monolayers at 37 °C was observed to increase with time (Fig. 3). This effect was strongly dependent on the type of surface modification. In the case of the non-modified liposomes and CP liposomes, the cellular association moderately increased by prolonging the incubation period from 1 to 2 h, and then no increase was observed up to 4 h. In contrast, the cellular association of WGA–CP liposomes markedly increased over the investigated time points with higher rate during the initial incubation period.

The effect of non-specific metabolic inhibitors on the cellular association of WGA–CP liposomes was investigated. Pre-incubation of the Caco-2 monolayers with 10 μg/ml chlorpromazine resulted in significant reduction in WGA–CP liposomes cellular association by approximately 27%, but did not completely abolish the active uptake of the particles when compared to the association level at 4 °C (Fig. 4). In contrast, the effect of 1 μg/ml filipin on the cellular association of the liposomes was not significantly different ($P > 0.05$). The effect of 10-fold excess of free WGA on the cellular uptake of WGA–CP liposomes was also investigated. As observed in Fig. 4, the total amount of cell associated liposomes was markedly reduced by approximately 44.2%. This competitive inhibition effect mediated by free WGA confirms the presence of a specific mechanism involved in the active binding and uptake of the WGA–CP liposomes.

For visualization of the role of WGA in cellular association and uptake of the liposomal carrier, the liposomes were loaded with DiI (red) and surface modified with FITC–WGA–CP (green). After incubation of this liposomal formulation with Caco-2 cell suspension for 1 h at 37 °C, dual channel confocal images were captured

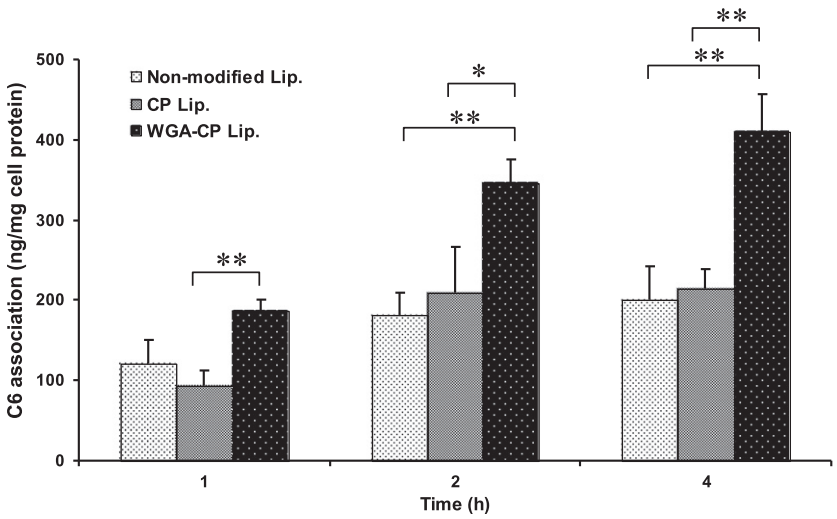

Fig. 3. Effect of incubation period on liposomal association with Caco-2 cell monolayers at 37 °C (mean ± SD, $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).

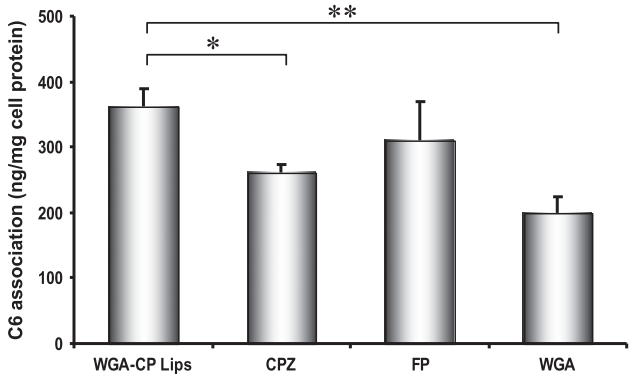

Fig. 4. Effect of pre-incubation with chlorpromazine (CPZ, 10 μg/ml), filipin (FP, 1 μg/ml), and free excess WGA (0.6 mg/ml) on Caco-2 association and uptake of WGA–CP liposomes at 37 °C (mean ± SD, $n = 4$, $^{*}P < 0.05$, $^{**}P < 0.01$).

in the *xy*-plane as well as in the *xz* and *yz* sections (Fig. 5). The green fluorescence of WGA and the red color of the liposomes were simultaneously observed both on the cell membrane and in the cytoplasmic compartment. This observation confirms the role of surface WGA in membrane binding and cellular uptake of the carrier system.

3.5. Intestinal bioadhesion and penetration of WGA–CP liposomes

The intestinal association and penetration of C6-loaded liposomes were visualized by CLSM. Segments from duodenum, jejunum, and ileum were isolated 2, 4, or 8 h after oral administration to rats and cryofixed prior to mounting for CLSM imaging. Images were captured on both the mucosal and basolateral sides of the intestinal membrane to evaluate the extent of mucosal penetration. Fig. 6 shows representative confocal images of rat's small intestine after intragastric administration of the fluorescent liposomes, and complete images are shown in Fig. S1 (supplementary data). The observed fluorescence on the intestinal membranes of rats received WGA–CP liposomes was more intense and prolonged than that of CP liposomes and non-modified liposomes groups. Moreover, fluorescence was clearly identified in the duodenal and jejunal segments of the WGA–CP group and could be detected deeply on the basolateral side of the intestinal membranes. These

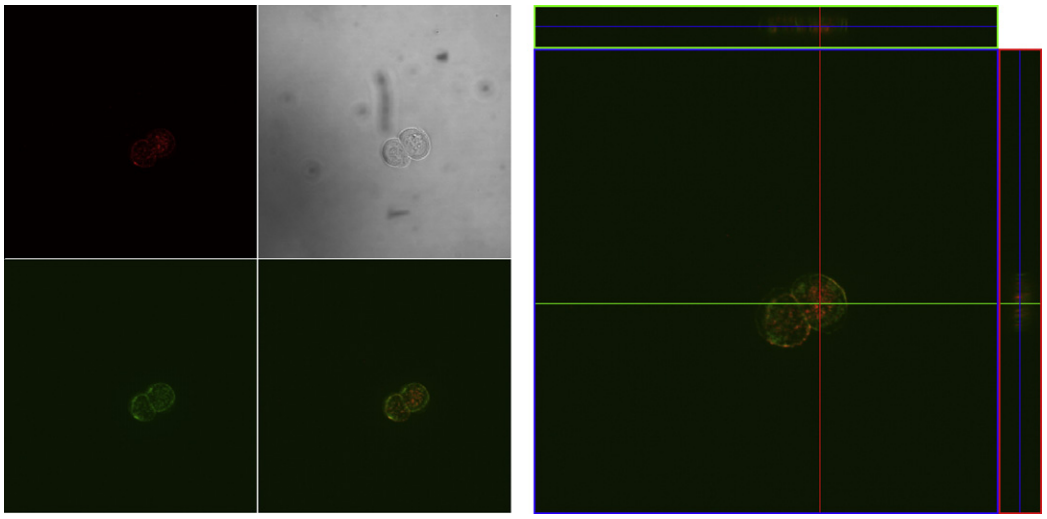

Fig. 5. Representative CLSM image of single Caco-2 cells after 1-h incubation period with WGA–CP liposomes. Red; DiI-labelled liposomes, green; FITC–WGA, yellow; merged.

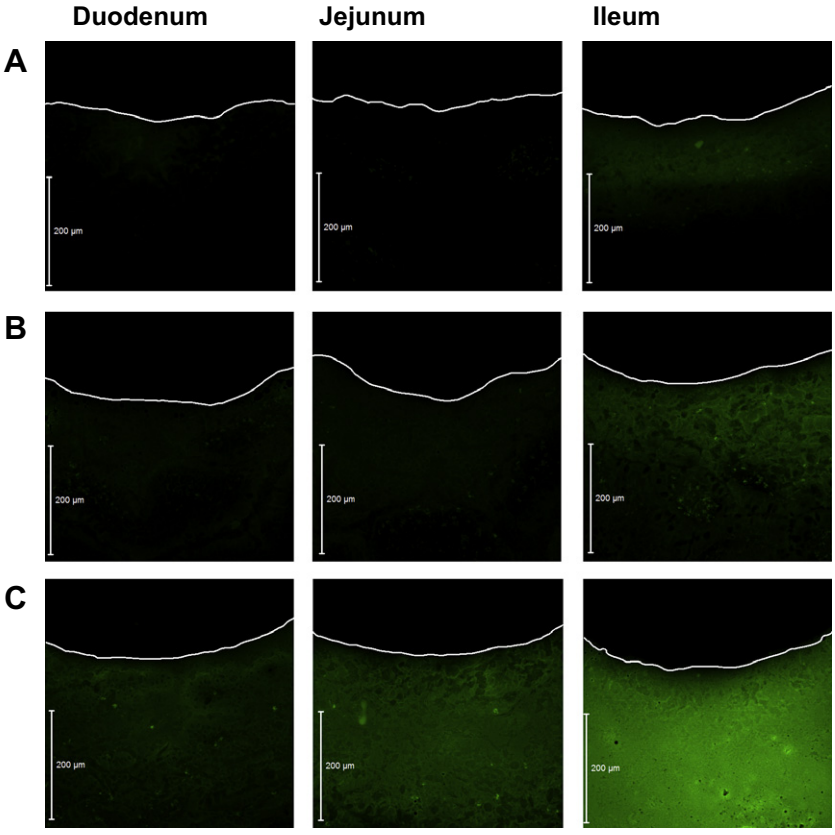

Fig. 6. Representative CLSM images for the bioadhesion and intestinal penetration of C6-loaded liposomes 4 h after intragastric administration to rats. (A) Non-modified liposomes, (B) CP liposomes, (C) WGA–CP liposomes.

results indicate the strong intestinal bioadhesion and penetration behavior of WGA–CP liposomes. Further evidence on the role of WGA in the bioadhesion and uptake of the liposomes was identified by dual channel CLSM (Fig. 7). The green fluorescence of FITC–WGA and the red dye of the liposomes were simultaneously recorded as previously observed with Caco-2 cells. The double-stained liposomes were clearly visualized deep inside the mucosal membrane for at least 200 µm.

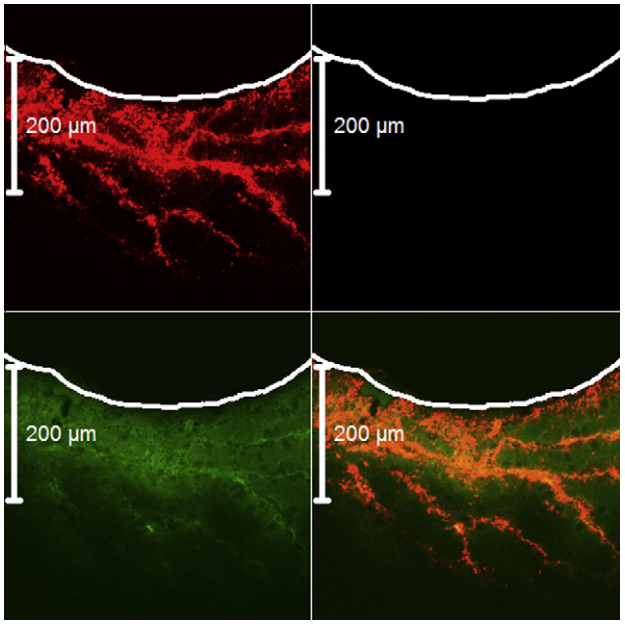

Fig. 7. Representative CLSM image of rat's ileal tissue 2 h after intragastric administration of WGA–CP liposomes. Red; DiI-labelled liposomes, green; FITC–WGA, yellow; merged.

3.6. Oral absorption of calcitonin

The ability of WGA–CP liposomes to enhance the systemic delivery of peptide drugs after peroral administration was investigated using calcitonin as a model peptide. Calcitonin was loaded into the liposomes during the step of lipid film hydration. The entrapment efficiency of calcitonin into the non-modified SUV liposomes was 90.9 ± 2.1%. Fig. 8 shows the percent reduction in blood calcemia and area above the plasma calcium concentration–time curve (AAC) after oral administration of the different liposomal formulations to rats. Results suggest that WGA–CP modified liposomes were more efficient in enhancing the absorption of the entrapped peptide. The therapeutic efficacy of calcitonin was significantly enhanced and prolonged when administered with WGA-functionalized liposomes. Comparison of the AAC values can provide a quantitative evaluation of the therapeutic efficiency of the different formulations with regard to reduction in blood calcemia. Surface modification of liposomes with WGA–CP resulted in more than 20- and 3-fold increase in the pharmacological efficacy of the entrapped calcitonin when compared to non-modified and CP liposomes, respectively.

4. Discussion

Enzymatic degradation and poor membrane permeability are the most common obstacles towards efficacious systemic delivery

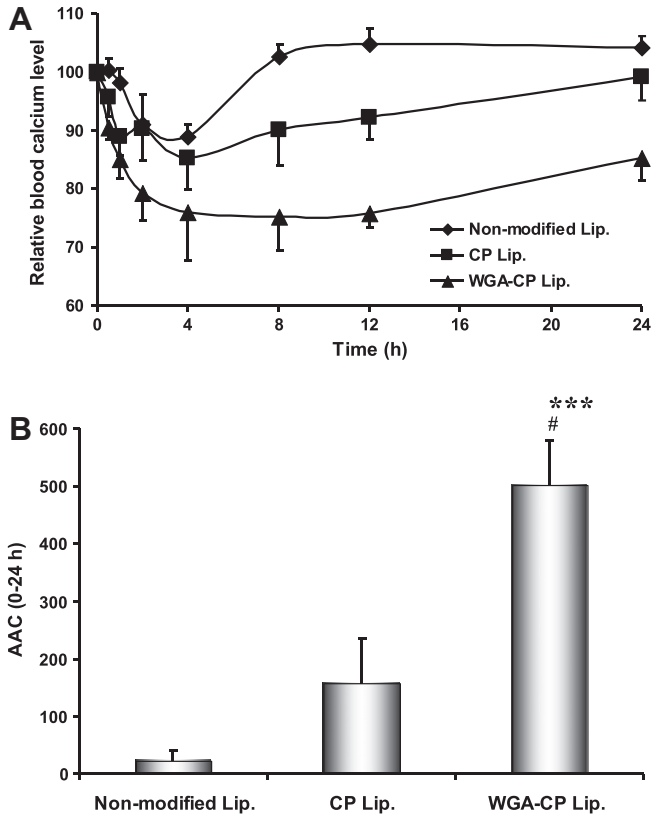

Fig. 8. Pharmacological effect of calcitonin-loaded liposomes (calcitonin dose; 20 μg/rat) after intragastric administration to rats. (A) Relative blood calcium level–time curve, (B) area above the blood calcium curve (AAC). Mean ± SD, $n = 4–6$ rats/group, $^{***}P < 0.001$ compared with non-modified liposomes, $^{\#}P < 0.05$ compared with CP liposomes.

of peptide and protein therapeutics after peroral administration. The use of particulate carriers and specific epithelial membrane recognizing ligands could be a promising approach to overcome these obstacles. Because of their biological compatibility, liposomal carriers have been considered as one of the most promising nanoparticle technologies for the delivery of therapeutic drugs, proteins, and genes. The encapsulation of the therapeutic agent within the liposomal core can provide sufficient protection against enzymatic detection and degradation [3]. In addition, modification of the liposomal surface has been recognized as a powerful approach to control liposome properties and biological behavior. It was suggested that coating of the liposomal surface with multifunctional polymers, such as chitosans and polyacrylates, significantly improves the liposomal retention on the intestinal mucosa by their mucoadhesive properties. Moreover, by virtue of their permeation enhancing and/or enzyme inhibiting effects, these polymers could further improve the systemic absorption of the entrapped peptide after peroral administration [16,17].

Lectins, including WGA, have been well recognized as powerful ligands that facilitate the cellular association and uptake of macromolecules and nanocarriers. They are capable of identifying and specifically binding to glycosylated components of the mammalian mucosa, either on the epithelial cells or in the mucus layer [14,18]. The cytoadhesive and cytopenetrative properties of lectins have been characterized using various cell culture and animal models [14]. Immobilization of lectins onto the surface of polystyrene nanoparticles led to a systemic uptake of 23% of the dose after oral administration to rats [19]. Using Caco-2 cell model, Russell-Jones et al. demonstrated the translocation of WGA-modified nanoparticles across the cell monolayers [20]. It was also observed that immobilization of WGA considerably enhanced the cellular binding as well as the uptake of PLGA nanoparticles [11]. In the present study, the effect of surface modification of submicron-sized liposomes with WGA–CP conjugate on their in vitro and in vivo behavior was evaluated.

Conjugation of WGA to the carboxylic functional groups of CP was accomplished by the carbodiimide coupling method. The amount of WGA chemically conjugated to CP was approximately 11.3 mg per 100 mg of the polymer conjugate with a conjugation efficiency of 60.4%. These values are significantly higher than that achieved in the case of WGA grafting onto PLGA [21,22]. Carbopol is a polyacrylate derivative composed of repeated carboxyvinyl units. Unlike PLGA, CP has abundant number of carboxylic groups per molecule that could be conjugated with amine compounds via amide linkage.

The receptor binding fraction of lectins represents a comparatively small part of the molecule, and the major part of the glycoprotein is not involved in the binding process [13]. However, chemical conjugation of lectins to polymers or drugs could partially or completely destroy their active binding sites. The ability of lectins to agglutinate erythrocytes has been regarded as a convenient method to measure their biological binding activity [19]. In the case of WGA–CP conjugate, haemagglutination was clearly observed even at very low concentrations of the conjugate (0.1 mg/ml). Moreover, the agglutination behavior after incubation of blood cells with WGA–CP was quite similar to that of the corresponding concentration of free WGA. These results confirm the ability of the conjugated WGA to efficiently associate with its specific cell membrane receptors and to retain its biological activity.

The results of the cytotoxicity study revealed the absence of any toxic effect of the prepared conjugate on Caco-2 monolayers up to a concentration of 4 mg/ml. Higher concentrations of CP or WGA–CP resulted in detachment of the cell monolayers during the washing steps, probably due to the high viscosity of the polymer solutions. Considering that intact intestinal membranes have a recovery mechanism from trauma and a protective mucus layer, which are absent in cell cultures, a higher safety profile of the prepared conjugate should be anticipated after oral administration. Lectins from plant origin, such as WGA, constitute an important part of the regular diet of many mammalians. It is estimated that each kilogram of flour or cereals to contain about 300 mg of intact WGA without any evidence of exerting negative effects. Therefore, the damaging effect from using plant lectins to improve the mucosal delivery of drugs should be negligible. Considering CP and other polyacrylate derivatives, they are approved by the FDA as safe polymers for oral formulation and have been shown to have no acute toxicity or cell damaging effects on Caco-2 cells [23].

The positively charged SUV liposomes were prepared using the thin film hydration method followed by probe sonication. The liposomal composition was selected based on our previous results on the stability of the produced liposomal formulations. Recently, we have shown that liposomes composed of DSPC have the highest rigidity and mechanical strength among all the investigated phospholipids [24]. DSPC exists in the gel state at room temperature, resulting in low fluidity of the liposomes. Moreover, liposomal rigidity was found to be dependent on the type of the positively charged additive, with higher stability for liposomes containing stearylamine [24].

The effect of surface modification with hydrophilic polymers, CP and WGA–CP, on liposomal association with Caco-2 cells was investigated. At first, we observed a significant enhancement in the association of liposomes with the cell monolayers by surface modification with WGA–CP. The effect was dependent on the amount of WGA on liposomal surface with the highest effect at WGA–CP concentration of 1 mg/ml. This concentration has previ-

ously resulted in marked haemagglutination of the blood erythrocytes. Therefore, a lectin-mediated association of the liposomal carrier with Caco-2 cells could be suggested. In general, cellular association of nanocarriers can be induced either by specific or non-specific adsorption of the carrier system onto the cell surface or by carrier internalization into the cells. While the association measured at 37 °C reflects the sum of internalized and surface-attached carrier, the cellular association at 4 °C reflects mere binding to the cell surface [13]. At 4 °C, the fluidity of the membrane and the metabolic activity of the cells are reduced; therefore, active uptake pathways for nanocarriers are minimal and surface binding is prevailing. Based on this assumption, more than 42.2% of the cell associated vesicles at 37 °C were internalized into Caco-2 cells by active processes in the case of WGA–CP liposomes. In addition, the amount of internalized WGA–CP liposomes was approximately 5.6- and 4.2-fold higher than that of non-modified and CP liposomes, respectively. The non-specific association observed at 4 °C can be attributed to the interaction between positively charged vesicles and negatively charged cell membrane in the case of non-modified liposomes, and by hydrophilic binding and polymer chain entanglement in the case of CP and WGA–CP liposomes [25,26]. Non-specific cell membrane association could be further promoted by the static condition of the horizontally arranged monolayers that might result in overestimation of membrane binding [11]. Considering the effect of incubation time, the cellular association of the non-modified and CP liposomes at 37 °C reached saturation after 2 h, indicating the presence of a limit for their non-specific membrane association. However, a continuous association of WGA–CP liposomes with Caco-2 monolayers was observed with time with higher rate during the initial 2 h. These results can be attributed to the balance between particles uptake inside the cells and membrane association.

The mechanism involved in the active uptake of WGA–CP liposomes was revealed by pre-incubation of Caco-2 monolayers with non-specific and specific inhibitors. Chlorpromazine and filipin are known to metabolically inhibit clathrin- and caveolin-mediated endocytosis processes, respectively [27]. Chlorpromazine, but not filipin, could significantly reduce the Caco-2 association of WGA–CP liposomes, but did not completely abolish the active uptake process. These results indicate that the active uptake of WGA–CP liposomes inside Caco-2 cells occurs, in part, by the formation of clathrin-coated vesicles as a result of surface membrane association. Additionally, the involvement of WGA-specific binding receptors in the cellular uptake was investigated by pre-incubation of the monolayers with 10-fold excess of free WGA. The latter is expected to compete with WGA on the liposomal surface for the active binding sites on the cell membrane. Using fluorescently labelled WGA, Wirth et al. have previously reported that all the binding sites available for WGA at the surface membrane of Caco-2 cells are occupied within 10 min of incubation [28]. In the current study, pre-incubation of the cell monolayers with excess of free WGA resulted in approximately 44.2% reduction in the total cellular association of liposomes. This competitive inhibition effect mediated by free WGA confirms the presence of a specific mechanism involved in the active binding and uptake of the WGA–CP liposomes. This conclusion was further supported by confocal imaging of the association of the double-stained liposomes with single Caco-2 cells. The obtained images clearly identify the role of WGA in the membrane association and intracellular uptake of the liposomal carrier.

According to the data in literature, lectin-mediated drug delivery should be regarded as a promising strategy to facilitate the oral absorption of poorly permeable drugs. Recently, we have shown that multilamellar liposomes (5- to 6-μm diameter) surface modified with WGA–CP could effectively adhere to the small intestine of rats for prolonged periods [4]. Due to their large particle size, the membrane adhesion of MLVs is presumably mediated by lectin binding to the mucus layer and/or epithelial surface. In the current study, the presence of WGA–CP on the surface of submicron-sized liposomes resulted in significant and prolonged surface association and deep penetration in the mucosal tissues. The small particles in the size range of 200 nm have the ability to transverse the epithelial membrane by virtue of lectin binding to its specific membrane receptors. The fact that lectin binding to the glycoprotein components of the mucus layer could reduce their binding capacity to the epithelial membrane has been identified [14]. Due to the continuous and rapid turnover of the mucus layer, lectins are expected to suffer, at least partially, from premature inactivation by shed off mucus [29]. However, the mucin content of wet GI mucus is only 0.5–4% by weight [30], and lectin binding to mucin was found to be saturable and reversible [29]. Therefore, the ratio between mucin and lectin content in formulation might be a crucial factor for lectin-epithelial cell association due to initial saturation of the mucus. In the case of WGA–CP liposomes, the use of a high surface amount of lectin conjugated to a mucoadhesive polymer seems advantageous. Mucin binding to WGA and CP could be regarded as an initial step that is followed by partitioning of the delivery system to the cell membrane receptors. In addition, uptake of the delivery system by the intestinal enterocytes could balance the equilibrium towards cytoadhesion rather than mucoadhesion.

The usefulness of the proposed WGA–CP liposomes in the peroral delivery of therapeutic peptides was evaluated using calcitonin as a model peptide. The pharmacological efficacy of the entrapped drug was significantly enhanced by more than 20-fold ($P < 0.001$) and 3-fold ($P < 0.05$) when compared to non-modified and CP liposomes, respectively. The reason for such a pronounced effect can be directly related to the intestinal adhesive and penetrative characteristics of lectins. WGA on the liposomal surface triggered the interaction of the liposomes and the intestinal membrane via its strong and specific association with glycoprotein receptors on the cell surface [11,13,21]. This interaction could provide a higher concentration of the drug either in direct contact with the cell surface or inside the cells for prolonged periods. In addition, polyacrylate polymers, including carbopols, are well known for their non-specific mucoadhesive properties mediated by physical chain entanglement and hydrogen bonding [25,26]. They are also reported to exhibit a strong inhibitory effect towards the proteolytic action of trypsin [31] and to display a paracellular permeation enhancing effect by transiently opening the tight junctions between epithelial cells [23]. These properties could explain the higher efficacy of CP liposomes in enhancing the oral absorption of calcitonin when compared to the non-modified carrier.

5. Conclusion

A nanoparticulate system that combines the specific membrane binding activities of lectins and the non-specific bioadhesive properties of polyacrylates was proposed to facilitate the intestinal membrane transport of peptide drugs. The carrier is based on surface modification of submicron-sized liposomes with CP conjugated to a high concentration of WGA. In vitro studies in cell cultures and in vivo animal studies revealed superior cellular association and membrane uptake of the carrier mediated by active binding of WGA to its membrane-specific receptors. In vivo studies also confirmed the ability of the delivery system to penetrate the intestinal barrier and enhance the systemic delivery of the entrapped peptide. All in all, surface modification of particulate carriers with the novel WGA–CP appears to be a promising approach to improve the mucosal uptake of peptides and proteins either for local immunization or systemic therapeutic purposes.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2010.12.008.

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