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International Journal of Pharmaceutics 191 (1999) 25–32

international journal of pharmaceutics

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# Preparation of *Ulex europaeus* lectin-gliadin nanoparticle conjugates and their interaction with gastrointestinal mucus

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Received 15 March 1999; received in revised form 2 July 1999; accepted 8 July 1999

**Abstract**

One approach to improve the bioavailability and efficiency of drugs consists of the association of a ligand (i.e. lectins), showing affinity for biological structures located on the mucosa surfaces, to nanoparticulate drug delivery systems. In this context, *Ulex europaeus* lectin-gliadin nanoparticle conjugates (UE-GNP) were prepared with the aim of evaluating their in vitro bioadhesive properties. The lectin was fixed by a covalent procedure to gliadin nanoparticles by a two-stage carbodiimide method. Typically, the amount of bound lectin was calculated to be $\sim 15$ μg lectin/mg nanoparticle, which represented a coupling efficiency of $\sim 16\%$ of the initial lectin concentration. In addition , the activity of these conjugates was tested with bovine submaxillary gland mucin (BSM) and the level of binding to this mucin was always much greater with UE-GNP than with controls (gliadin nanoparticles). However, the presence of 50 μmol fucose, which is the reported specific sugar for *U. europaeus* lectin, specifically inhibited the activity of these conjugates and, therefore, the UE-GNP binding to BSM was attenuated by 70%. These results clearly showed that the activity and specificity of *U. europaeus* lectin was preserved after covalent coupling to these biodegradable carriers. 

*Keywords:* Gliadin; Lectin; Nanoparticles; Mucin; Bioadhesion

## 1. Introduction

Optimum contact between the drug delivery system and the target biological surface could be useful for increasing drug absorption or to enhance drug bioavailability. Ideally, these dosage forms should be able to form strong interactions with epithelial surfaces, preventing displacement from the site of adhesion, similar to the interactions developed by some microorganisms. The process of microorganism attachment to a biological surface is mediated by the physico-chemical properties of the interface (Olsson et al., 1976) and by bacterial cell surface adhesins (such as fimbria, pili, or flagella) with specific carbohydrate-binding capacity.

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0378-5173/99/$ - see front matter 
PII: S0378-5173(99)00232-X

In this respect, the preparation of conjugates between colloidal carriers (i.e. liposomes and nanoparticles) and ligands for pharmaceutical purposes can offer some advantages either in specific drug delivery applications (i.e. monoclonal antibodies) or to increase the residence time of the pharmaceutical form (i.e. lectins).

Lectins comprise a structurally very diverse class of proteins, found in organisms ranging from viruses and plants to humans (Barondes et al., 1994), characterised by their ability to bind carbohydrates with considerable specificity. This property suggests that they play a role in biological-recognition events (Rini, 1995). Moreover, they have a high affinity and a narrow range of specificity for defined sugar residues. Thus lectins can be classified into a small number of specificity groups (mannose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, L-fucose, and *N*-acetyl neuraminic acid) according to the monosaccharide which is the most effective inhibitor of the agglutination of erythrocytes or precipitation of carbohydrate-containing polymers by the lectin (Lis and Sharon, 1986). Lectins are widely employed for biological and medical applications, in areas as diverse as blood typing (Delbaere et al., 1990), mitogenic stimulation (Grillon et al., 1991), bacterial typing (Slifkin and Doyle, 1990), and bone-marrow transplantation (Lapidot et al., 1990).

Moreover, in recent years, lectins have been described as "second generation mucoadhesives" (Kompella and Lee, 1992). In mammalian mucosae, sugar residues are located either at the surface of epithelial cells or in the mucus layer formed by glycoproteins. In this context, lectins have been proposed as tools for enhancing drug delivery to the gastrointestinal tract (Naisbett and Woodley, 1995; Irache et al., 1996), ocular surfaces (Nicholls et al., 1993) and the oral cavity (Nantwi et al., 1997).

Therefore, the selection of an appropriate lectin to prepare conjugates formed by the covalent coupling of these ligands to nanoparticles (or other type of drug carrier) may be of interest for targeting residues or receptors located on the mucosa surface. In this way, the residence time of the drug delivery system (lectin conjugate) at the site of drug activity or absorption should be increased.

The aim of this work was to prepare, characterise and evaluate the in vitro binding activity to a biological model (bovine submaxillary gland mucin) of a mucosal delivery system formed by a biodegradable carrier (gliadin nanoparticles) and a ligand showing specific properties (*Ulex europaeus* lectin).

## 2. Materials and methods

### 2.1. Materials

*U. europaeus* agglutinin I (UE), bovine submaxillary gland mucin (BSM) and α-(L)-fucose were purchased from Sigma (St. Quentin-Fallavier, France). Synperonic PE/F 68 was purchased from I.C.I. (Kortenberg, Belgium). 1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride was supplied by Aldrich (St. Quentin-Fallavier, France). Sodium hydroxide, sodium chloride and other chemicals used were of reagent grade and obtained from Prolabo (Paris, France).

### 2.2. Gliadin isolation and purification

Gliadin was extracted from dried gluten powder. In brief, 50 g of gluten (from a common wheat flour, variety Hardi) were stirred in an ethanol/water mixture (70/30 v/v; gluten/solvent ratio: 1/10) for 4 h at 20°C. The suspension was centrifuged and supernatants were dialysed exhaustively first against water and then against 0.05 M acetic acid. Finally, gliadin was freeze-dried.

Analysis of the extracted gliadin fraction was carried out by reverse phase-HPLC (Wieser et al., 1994) and polyacrylamide gel electrophoresis at acid pH (Larré et al., 1991). In this way, the amount of protein in the gliadin freeze-dried extract was calculated to be ~85% w/w and the proportions of the different gliadin groups were 55% for w/w alpha- and beta-gliadins, 15% for w/w gamma-gliadin, and 15% for w/w omega-gliadin.

### 2.3. Preparation of UE lectin-gliadin nanoparticle conjugates (UE-GNP)

Firstly, gliadin nanoparticles (GNP) were prepared by a desolvation method previously described (Ezpeleta et al., 1996). Briefly, 100 mg of gliadin were dissolved in 20 ml of an ethanol/water phase (7:3 v/v and this solution was poured into 40 ml of a stirred physiological saline phase (NaCl 0.9% w/v in water) containing 0.5% w/v Synperonic PE/F 68. The organic solvent was then removed by evaporation under reduced pressure (Büchi RE-140, Switzerland). Gliadin nanoparticles prepared in this way were purified by centrifuging twice (Beckman J2-21M/E centrifuge equipped with a J 20.1 rotor) at 20 000 rpm for 15 min. The supernatants were removed and the pellets were resuspended in phosphate buffered saline (PBS; pH 7.4, ionic strength 0.15 M).

Secondly, UE lectin was covalently bound to gliadin nanoparticles by an appropriate modification of the two-stage carbodiimide method (Irache et al., 1994). In brief, the carboxylic groups on the gliadin nanoparticle surface were activated in PBS by addition of a variable amount of 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride. After removing the excess carbodiimide reagent, 250 μg of UE lectin were then added and coupling was carried out by overnight incubation at room temperature. The conjugates were centrifuged three times to remove the free lectin, and aliquots of the clear supernatants analysed by size exclusion HPLC. Finally, conjugates were resuspended in PBS containing 5% w/v of glycerol and 0.2% w/v sodium azide as preservative. All conjugates were stored at 4°C.

### 2.4. Determination of nanoparticle size and yield

The particle size of the conjugates was measured by photon correlation spectroscopy on a Coulter submicron particle analyzer N4MD (Coultronics, Margency, France). The nanoparticle concentration was determined, after digesting the gliadin nanoparticle suspension, by spectrophotometry on a Perkin Elmer Lambda 5 spectrophotometer.

### 2.5. Determination of lectin content

The amount of fixed lectins was determined by HPLC analysis. The HPLC system was equipped with a 110-A Beckman pump, a Rheodyne model 7125 as sample injector with a 20-μl loop, and a Beckman 166 UV detector operating at 280 nm. A Toso Haas (Interchim, France) QC-PACK G300 gel filtration column (150 mm × 7.8 mm i.d., 5 μm) was used at room temperature. The mobile phase was 0.1 M phosphate buffer (pH 6.7), with 0.05% sodium azide and 0.1 M sodium sulphate, at a flow rate of 1 ml/min.

Aliquots of the clear supernatants obtained from centrifugations during the removal of the free lectin were taken and 20 μl were injected. The quantity of lectin bound to the particles was calculated as the difference between the initially added lectin and the lectin which was recovered by centrifugation.

### 2.6. In vitro studies with BSM

The binding capacity of UE-GNP was determined by mixing 1 ml BSM solution in PBS (1 mg/ml) with the same volume of the conjugate suspensions (15 μg of bound lectin/ml) in PBS. After incubation, the samples were centrifuged for 10 min at 10 000 rpm, and aliquots of the supernatants were taken and analysed by size exclusion HPLC as described above. The amount of interacted or bound mucin was calculated as the difference between the reference incubation (mucin alone) and the BSM remaining in the supernatants of the samples. The reference incubation contained the same amount of mucin as the samples.

For competitive inhibition studies, the sugar α-(L)-fucose was added to the mucin preparations in PBS and binding studies were performed as described above.

## 3. Results

Under the chromatographic conditions used, UE presented a major peak at 4.81 min (molecular weight ~ 70 000) which was subsequently used

for quantitative analysis (Fig. 1A). Concerning the validation procedure of this technique, the standard curve was linear for the studied range of concentrations ($r > 0.997$) and passed through the origin. The reproducibility was determined to be 0.39% for the 500 μg UE lectin/ml PBS ($n = 6$), and 0.98% for the 50 μg/ml ($n = 6$). Moreover, the quantitation limit was calculated to be 5 μg/ml.

On the other hand, gliadin nanoparticles were prepared according to a desolvation method for macromolecules, which enabled us to obtain submicronic carriers with a yield close to 90% of the initial protein. The UE lectin attachment to these nanoparticles was assessed as a function of the carbodiimide reagent concentration (amount of carbodiimide reagent added to the nanoparticulate suspension) and the reaction time (incubation time between the carbodiimide reagent and the gliadin nanoparticles). All experiences were performed at neutral pH to prevent carbodiimide reagent degradation. However, in order to eliminate the non-covalently associated lectin, three centrifugation steps were considered necessary. After each centrifugation, supernatants were collected (for analysis by HPLC) and particles resuspended in PBS. It was observed that no further detachment was detected during the third centrifugation step.

The influence of the carbodiimide activation time on lectin attachment to the gliadin nanopar-

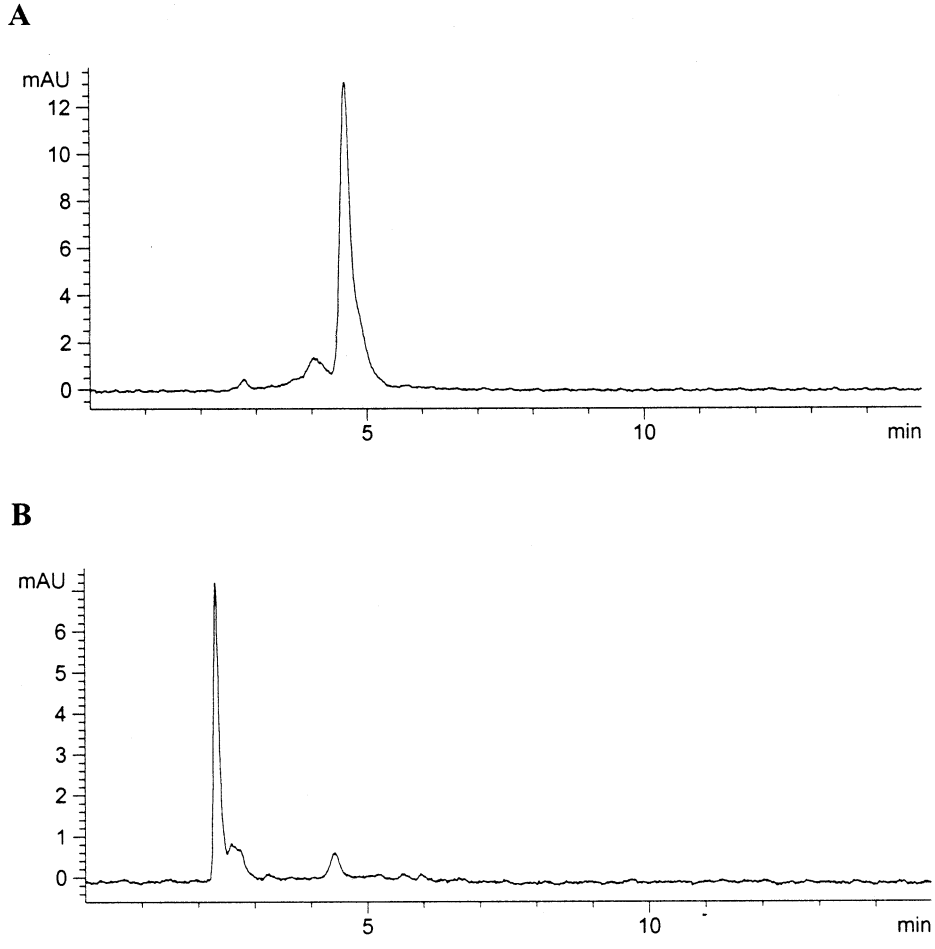


Fig. 1. Gel permeability HPLC. (A) Chromatogram of UE lectin (250 μg/ml). (B) Chromatogram of BSM in PBS (250 μg/ml).

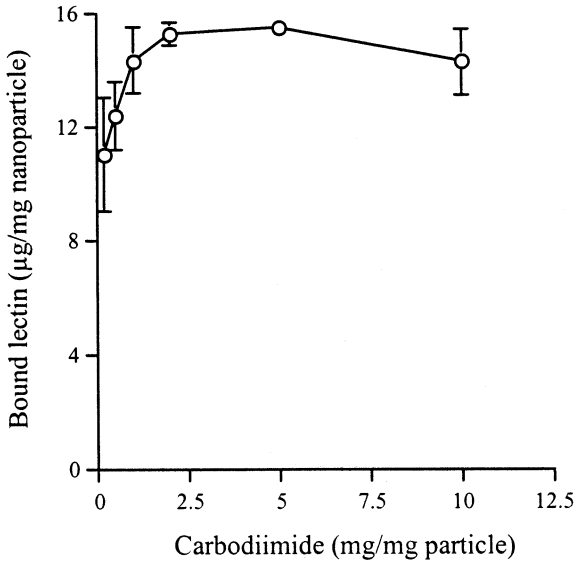


Fig. 2. Effect of increasing carbodiimide concentration on the activation step in the binding of lectin to gliadin nanoparticles. Experimental conditions: room temperature, reaction time = 3 h, PBS (pH 7.4), $n = 3–4$.

ticles was assessed at the concentration of 5 mg carbodiimide reagent per mg nanoparticles, and the ideal contact time between the reagent and the gliadin carriers was estimated to be 3 h (data not shown). Concerning the influence of the carbodiimide reagent concentration on the lectin attachment to gliadin nanoparticles, Fig. 2 shows the obtained results. After 3 h of incubation, ligand binding increased with the carbodiimide concentration. In this case, a plateau could be reached at a reagent concentration of $\sim 2$ mg carbodiimide/mg nanoparticles.

Under the experimental conditions described above (neutral pH, 3 h of incubation time between carbodiimide reagent and nanoparticles, and carbodiimide reagent concentration of 2 mg/mg nanoparticles), the UE lectin-gliadin nanoparticle conjugates (UE-GNP) typically showed an average particle size of $\sim 587 \pm 35$ nm (average polydispersity index = 0.054), and the amount of bound lectin was calculated to be $15.25 \pm 0.42$ μg lectin/mg nanoparticles. This value represented a lectin coupling efficiency of $\sim 16\%$ of the initial ligand concentration.

Fig. 3 shows the amounts of BSM which had bound to the UE-GNP and the control (unmodified gliadin nanoparticles, GNP) as a function of time. BSM by size exclusion HPLC analysis showed three peaks (Fig. 1B) and their total surface was used for quantitative analysis.

The equilibrium for UE-GNP interaction to BSM was reached after incubation for 60 min. The plateau levels, i.e. interacted BSM at 90 min was calculated to be $\sim 18\%$ bulk mucin. A mathematical model based on the Langmuir equation (Vincent et al., 1980) was used to determine the rate constant of interaction. Considering that the binding was irreversible, this rate was calculated to be $1.02 \times 10^{-3}$ (mg min/ml)$^{-1}$.

Fig. 4 shows the amounts of BSM which interacted with the UE-GNP or GNP (used as control) after an incubation time of 1 h. Experiments were carried out in the absence and in the presence of α-(L)-fucose, which is the reported specific sugar for UE lectin (Chai et al., 1992). At a fucose concentration of 50 μmol, the BSM binding to UE-GNP was attenuated by 70%.

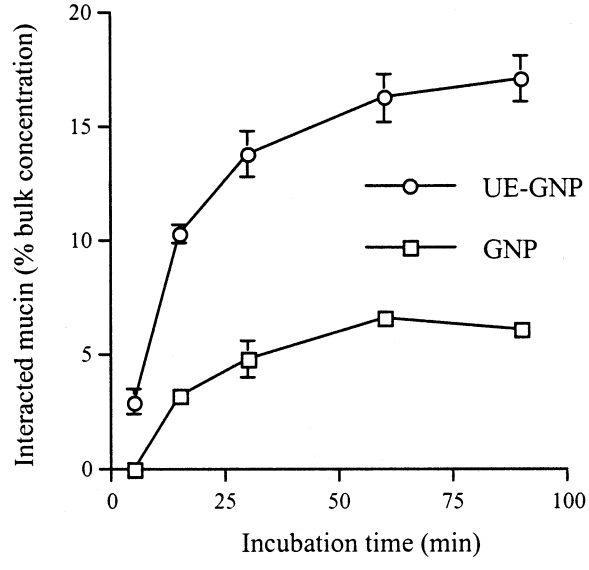


Fig. 3. Kinetics of BSM interaction with UE-GNP (○, 7.5 μg available lectin/ml incubation medium) and GNP (□, 0.5 mg/ml) used as controls. Experimental conditions: mucin bulk concentration = 250 μg/ml, room temperature, PBS (pH 7.4), $n = 4$.

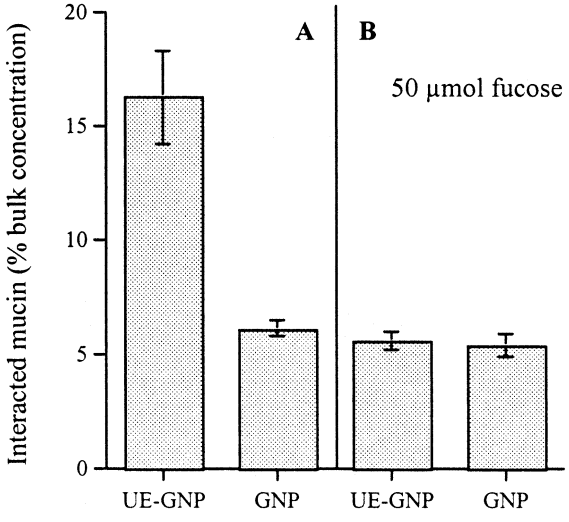


Fig. 4. BSM binding to UE-GNP (7.5 µg of bound lectin per ml incubation medium) and GNP (0.5 mg per ml) in suspensions without (A) or with (B) 50 µmol fucose. Experimental conditions: room temperature, incubation time = 1 h, PBS (pH 7.4), $n = 4$.

## 4. Discussion

The term gliadin defines a group of polymorphic proteins extracted from gluten which are soluble in ethanolic solutions and show a remarkably low solubility in water, except at extreme pH. Due to these physico-chemical properties, gliadin nanoparticles can be prepared, by a desolvation method for macromolecules, using environmentally acceptable solvents such as ethanol and water. These macromolecules showed a high capacity for loading lipophilic drugs and were stable without further chemical or physical cross-linking treatment (Ezpeleta et al., 1996).

In general, proteins produce biodegradable particles whose physico-chemical properties can be modulated by the cross-linking methods employed for their production. Furthermore, due to the presence of numerous functional groups (i.e. amino and carboxylic residues), proteins are excellent candidates for the preparation of conjugates, formed by the attachment of molecules capable of providing specificity to the surface of nanoparticles such as lectins. In our case, the lectin was fixed by the two-stage carbodiimide coupling technique. Carbodiimide involves the activation of carboxylic acid groups to give NH-activated groupings which can react with free amino groups of the ligand polypeptide chains (Olde Damink et al., 1996). In proteins, these carboxylic groups can be found on aspartic and glutamic acid residues. For gliadin, Asp and Glu only represent 21 and 71 groups per 1000 aminoacid residues, respectively (Ewart, 1983; He et al., 1992). In spite of gliadin having a very low proportion of these two aminoacids, it appears that the coupling reaction is efficient enough to provide an acceptable lectin binding.

In principle, it is possible to suppose that these bioadhesive devices can be used (i) for increasing the residence time of the administered drug within the gut, (ii) to improve the bioavailability and efficiency of therapies, and (iii) to develop new therapeutic strategies based on site specific delivery within the gut. Under this third purpose, the design and preparation of lectin conjugates can be of interest for different target objectives within the mucosa such as epithelial cells, absorptive windows, cancerous cells and Peyer's patches or gut associated lymphoid tissue (GALT).

Nevertheless, in spite of such potential, the development of such systems has been limited by the absence of polymeric materials able to prepare biodegradable particles with suitable functional groups for stable association with the chosen ligand. In this context, the use of gliadins permits the development of biodegradable and active lectin conjugates for the delivery of hydrophobic drugs. Moreover, these carriers have been proved effective in protecting the loaded drug and controlling its release (Ezpeleta et al., 1996).

As far as the amount of binding mucin was concerned, interactions were always much higher with UE-GNP than with control (Fig. 3). It appears that these conjugates show an important affinity for this mucin in spite of the fact that fucose only represents less than 4% of the carbohydrate chain of BSM (Honda and Suzuki, 1984). This fact may be explained by the BSM structures of the oligosaccharide chains linked to the polypeptide backbone. In fact, BSM includes the following specific hexasaccharide structure: GlcNAc(β1-6)- [GalNAc(α1-3)- (Fuc(α1-2)]- Gal(β1-

4)- GlcNAc(β1-3)- *N*-acetylgalactosa minitol (Chai et al., 1992). Furthermore, *U. europaeus* lectin I is specific for oligosaccharides containing fucose with the following structure: -Fuc(α1-2)-Gal(β1-4)- GlcNAc- (Gohier et al., 1996). In the human body, this last specific oligosaccharide structure can at least be found in cecal M-cells (Gebert and Posselt, 1997) and on the surface of stomach cells from antral and body regions (Falk et al., 1993).

The inhibition studies with fucose confirmed the reported specific characteristics of *U. europaeus* lectin I for α-(L)-fucose. The interactions between conjugates and mucins dramatically decreased when the competing sugar was added (Fig. 4). Under these conditions, the interaction was approximately similar to that obtained with control particles. Therefore, these results clearly suggested that the lectin remained active after its covalent coupling to nanoparticles, and UE-GNP kept the same binding characteristics with fucose residues as the lectin.

In conclusion, this study has shown that gliadin nanoparticles are a good substrate for binding UE lectin. The activity of *U. europaeus* lectin, as was demonstrated by inhibition studies, was preserved after covalent coupling to these biodegradable carriers. Therefore, the use of these lectin-nanoparticle conjugates could be of interest for improving the efficiency of interactions between the pharmaceutical dosage form and different biological structures located on the mucosa.

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