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Preparation of lectin-vicilin nanoparticle conjugates using the carbodiimide coupling technique

Isabel Ezpeleta[a], Juan M. Irache[a,*], Serge Stainmesse[a], Christiane Chabenat[b], Jacques Gueguen[c], Anne-Marie Orecchioni[a]

[a]*Département de Pharmacie Galénique, Laboratoire Pharmacochimie et Biopharmacie (EA DRED 1295), Université de Rouen, B.P. 97, 76803 Saint Etienne du Rouvray, Cedex, France*

[b]*Département de Chimie Analytique, Université de Rouen, B.P. 97, 76803 Saint Etienne du Rouvray, Cedex, France*

[c]*INRA, Laboratoire de Biochimie et Technologie des Proteines, rue de la Géraudière, 44026 Nantes, Cedex, France*

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Abstract

This work describes the preparation and the in vitro activity of conjugates formed by *Ulex europaeus* lectin (UE lectin) and vicilin (storage protein from *Pisum sativum*) nanoparticles. The lectin was fixed by coupling its amino groups to carbodiimide-activated carboxylic groups on the vicilin nanoparticles. The influence of the carbodiimide concentration and the reaction time was studied. Typically, the amount of bound lectin was calculated to be about 30 μg lectin/mg nanoparticles, which corresponded to a lectin coupling efficiency of about 21%. Furthermore, the activity and the specificity of these conjugates was tested with bovine submaxillary gland mucin (BSM). When the experiences were carried out in the absence of L-fucose (specific sugar for UE lectin), the UE lectin-vicilin nanoparticle conjugates showed three times more interaction with mucin than the control (vicilin nanoparticles). Moreover, the specificity of the lectin was maintained after coupling to vicilin nanoparticles because the presence of L-fucose inhibited the interactions between conjugates and mucin.

Keywords: Vicilin; Nanoparticles; Lectin; Mucin; Conjugates; Carbodiimide

1. Introduction

Microparticles, nanoparticles and liposomes are widely employed in various areas of life sciences, including as drug delivery systems and sustained release systems. For some of these applications, the surface of these carriers (particles and lipo-

* Corresponding author. Tel.: +33 35663875; fax: +33 35665575.

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somes) must be modified with various functional molecules such as antibodies (Gregoriadis, 1982; Weinstein and Leserman, 1984; Al-Abdulla et al., 1989), lectins (Lehr et al., 1992; Irache et al., 1994), carbohydrates (Kobayashi and Sumimoto, 1983; Maruyama et al., 1994) and other biological ligands in order to recognize or bind particular molecules.

On the other hand, the association of ligands with liposomes or nanoparticles can be achieved by different procedures, including covalent linkage and adsorption processes. Ideally, the ligand should be conjugated to particles through a covalent linkage, which is more stable than adsorption procedures, without affecting its specificity. Non-covalent attachment relies on a fortuitous process. Further, it is difficult to control the amount of ligand which is associated and this process is useful only for those proteins that will associate non-specifically (Betageri et al., 1993).

Different techniques for covalent attachment may be envisaged, depending on the principal functional groups located on the carrier surface. The most widely used methods of ligand coupling are the use of cyanogen bromide (Al-Abdulla et al., 1989; Dolinnaya et al., 1991) and periodate (Boorsma and Streefkerk, 1979; Tijssen and Kurstack, 1984) for hydroxyl groups, glutaraldehyde (Avrameas and Guilbert, 1972; Otto et al., 1973) and ethylene glycol diglycidyl ether (Sano et al., 1993) for amino groups, and carbodiimide for carboxylic groups (Molday et al., 1975; Shenoy et al., 1992).

With periodate and glutaraldehyde, coupling occurs via the formation of a Schiff base between an amino group and an aldehyde function. With the first method, aldehydes are generated when periodate oxidizes the sugars bound to the peptide chain, while in the second glutaraldehyde supplies the aldehydes. Carbodiimide involves the activation of carboxylic acid groups to give NH-activated carboxylic acid groups which can react with free amino groups of the ligand polypeptide chains (Olde Damink et al., 1996). Moreover, glutaraldehyde and carbodiimide coupling may be done in one or two steps: in the one-step process, all the components of the reaction are present at the same time, while in the two-step method, the functional groups on the carrier surface (amino or carboxylic residues) are first activated by the appropriate reagent, the excess of which is removed before ligand attachment.

Due to the presence of numerous functional groups for the coupling of drugs, proteins are excellent candidates for prodrug synthesis (Vermeersch and Remon, 1994) or for conjugation with molecules capable of providing specificity to a drug delivery system. For this reason we have chosen nanoparticles prepared from vicilin (a storage vegetal protein from *Pisum sativum*) as the drug carrier in a complex system in which the particles are combined with molecules which have a specificity for sugars, such as lectins.

The name lectin originally described plant extracts capable of agglutinating red cells (Boyd and Shapleigh, 1954). Nevertheless, nowadays a lectin is defined as a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates (Goldstein et al., 1980). Furthermore, the ability of lectins to interact, via receptors, with different types of animal cells is inhibited by an excess of simple sugars, usually monosaccharides. It is assumed that these sugars represent the binding site for the lectin on the cell surface.

The aim of this work was to optimize the preparation and to test in vitro the activity of a delivery system formed by a carrier (vicilin nanoparticles) and a ligand showing specific properties (*Ulex europaeus* lectin).

2. Materials and methods

2.1. Materials

Ulex europaeus agglutinin I (UE lectin), bovine submaxillary gland mucin (BSM), α-(L)-fucose, and glutaraldehyde were purchased from Sigma (St. Quentin-Fallavier, France). 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. Methods

2.2.1. Vicilin isolation

Vicilin samples (molecular weight 160 kDa) were isolated and purified from selected pea seeds (*Pisum sativum*, var. Amino) by a chromatographic procedure using successive ion exchange and gel filtration steps as described elsewhere (Gueguen et al., 1984; Larré and Gueguen, 1986). Briefly, the crude protein extract was fractionated on DEAE Sepharose CL 6B and the vicilin fraction was eluted from a preparative column (Pharmacia K100/45), with 0.1 M NaCl in sodium phosphate-citrate buffer (0.1 M, pH 7) at a flow rate of 40 ml/h cm^2. This fraction was concentrated by ultrafiltration and purified by gel filtration on a column packed with Ultrogel ACA 34. Finally, vicilin was desalted through Trisacryl GF05 and freeze-dried.

2.2.2. Vicilin nanoparticle preparation

Vicilin nanoparticles were prepared according to the procedure described elsewhere (Ezpeleta et al., 1996). In brief, an aqueous solution of vicilin (adjusted to pH 9 with NaOH 0.01 N) was mixed with a constantly stirred phosphate buffer solution (pH 6.4, ionic strength 0.10 M). The coacervates thus obtained were hardened by chemical cross-linkage with glutaraldehyde (to give 3 mg glutaraldehyde/mg protein or 52.1 μmol glutaraldehyde/μmol lysine) for 2 h at room temperature. Vicilin nanoparticles were purified three times by centrifugation at 20 000 rpm for 15 min (Beckman J2-21 M/E centrifuge equipped with a J 20.1 rotor), and the particles were finally resuspended in phosphate buffered saline (PBS; pH 7.4, ionic strength 0.15 M, 0.2% sodium azide as preservative). Under these conditions, the resulting nanoparticles had an average diameter of approximative 660 nm.

The size of vicilin nanoparticles was measured by photon correlation spectroscopy on a Coulter® submicron particle analyzer N4MD (Coultronics, Margency, France), whereas the concentration was determined by spectroscopy (Ezpeleta et al., 1996), after digestion of samples with NaOH, on a Spectronic 601 UV-Vis spectrophotometer (Bioblock Scientific, Illkirch, France).

2.2.3. Preparation of UE lectin-vicilin nanoparticle conjugates

UE lectin was covalently bound to vicilin nanoparticles by an appropriate modification of the two-stage carbodiimide method described previously (Irache et al., 1994). Briefly, a variable amount of 1-(3-dimethylamino propyl)-3-ethyl carbodiimide hydrochloride was added to a vicilin nanoparticle suspension in PBS, which was then stirred at room temperature for up to 6 h. After removing the unreacted carbodiimide, 300 μg of UE lectin were added and coupling was carried out by incubation overnight at room temperature. The conjugates were centrifuged three times to remove the free lectin. Then, UE lectin-vicilin nanoparticle conjugates were resuspended in a PBS solution containing 5% w/v of glycerol and 0.2% w/v sodium azide as preservative. All conjugates obtained were stored at 4°C.

2.2.4. Measurement of lectin by HPLC

The amount of fixed lectins was determined by high performance liquid chromatography (HPLC) analysis. The system consisted of an isocratic solvent delivery pump (110 A Beckman pump), a sample injector (Rheodyne model 7125) equipped with a 20-μl loop, and a variable wavelength UV detector (Beckman model 166) 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.

In order to determine the molecular weight of UE lectin, the column was firstly calibrated with appropriate molecular weight markers (Kit No. MW-GF-200, Sigma). Moreover, a standard curve for UE lectin was drawn between 25 and 500 μg lectin/ml PBS. Samples were always analyzed by the external standard method.

Aliquots of the clear supernatants obtained from centrifugation 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.

2.2.5. In vitro studies with bovine submaxillary mucin (BSM)

The in vitro biological activity of the conjugates were determining by mixing 1 ml of BSM solutions in PBS (0.5 mg/ml) with the same volume of suspensions of the UE lectin-vicilin nanoparticle conjugates (30 μg of bound lectin/ml) in PBS. After incubation for 60 min, the samples were centrifuged for 10 min at 10 000 rpm, aliquots of the supernatants were taken, and 20 μl was injected into the HPLC system. The amount of interacted BSM was calculated as the difference between the total (reference system) and the remaining BSM in the clear supernatant. The reference systems consisted of the same amount of BSM as in the samples (250 μg/ml PBS), centrifuged as described above.

For specificity studies, α-L-fucose was added to the BSM bulk solutions in PBS and the interaction studies with the UE lectin-vicilin nanoparticle conjugates were performed as described above. The fucose bulk concentration in the BSM bulk solution was 100 mM.

3. Results and discussion

3.1. UE lectin analysis

Ulex Europaeus seed extracts give at least two different lectins: Ulex I which is inhibited by L-fucose, and Ulex II, which is specific for di-*N*-acetylchitobiose (Matsumoto and Osawa, 1969). In our case, the lectin corresponded to type I and was therefore specific for L-fucose.

Under the chromatographic conditions used, *Ulex europaeus* lectin I (UE lectin) presented a major peak at 4.81 min, which was subsequently used for quantitative analysis (Fig. 1). The molecular weight of UE lectin was estimated as approximately 70 000, according to the retention time on the calibrated column. Furthermore, the validation procedure of the gel filtration HPLC technique included linearity, reproducibility and quantitation limit. Thus, the standard curve was linear for the studied range of concentrations and passed through the origin, with a correlation coefficient of 0.997. 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. Finally, no modification of the retention conditions of the UE lectin peak was observed during the experimental period.

3.2. Influence of carbodiimide on UE lectin fixation

The UE lectin attachment to vicilin particles 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 vicilin nanoparticles). All experiences were performed at neutral pH because carbodiimide reagents rapidly lose their activity in aqueous media at acidic pH, producing the corresponding urea derivative

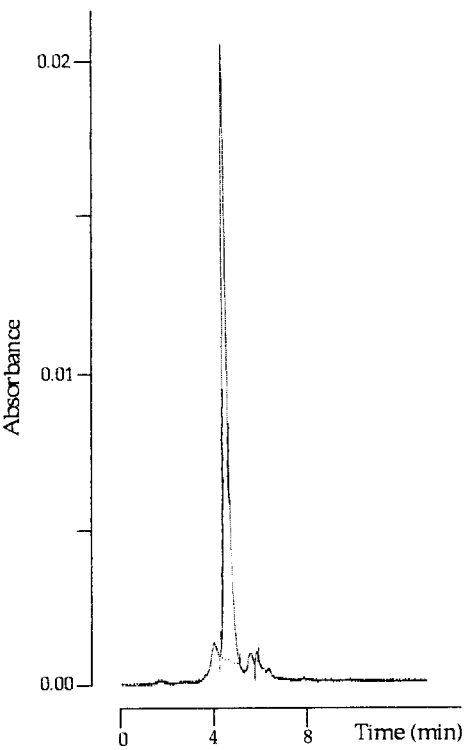

Fig. 1. Gel permeability HPLC. Typical chromatogram of UE lectin (250 μg/ml).

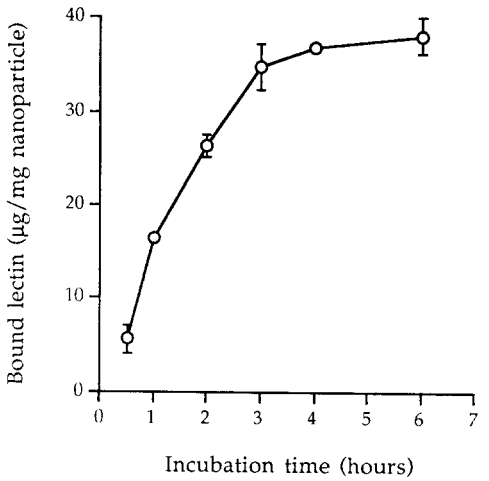

Fig. 2. Effect of increasing carbodiimide activation time on the binding of lectin to vicilin nanoparticles. Experimental conditions: room temperature, carbodiimide reagent bulk concentration = 1.25 mg/mg nanoparticles, pH 7.4 (PBS), $n = 3$–4.

(Nakajima and Ikada, 1995). Moreover, preliminary experiences had shown that vicilin particles are not stable under basic conditions (data not shown).

On the other hand, 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 vicilin nanoparticles was assessed at the concentration of 1.25 mg carbodiimide reagent per mg nanoparticles (Fig. 2). The curve indicates an increase in lectin binding with the time. Moreover, an equilibrium was reached after incubation for at least 3 h. The plateau level, i.e. lectin bound at 6 h, represented an UE lectin fixation efficiency of about 24% of the initial ligand bulk concentration.

Fig. 3 shows the influence of the carbodiimide reagent concentration on the lectin attachment to vicilin nanoparticles after 3 h of incubation. Similarly, ligand binding increased with the carbodiimide concentration. In this case, a plateau could be reached at a reagent concentration of about 0.7 mg carbodiimide/mg vicilin nanoparticles. Moreover, in experiences carried out in the absence of carbodiimide, it was noted that a small amount of lectin remained bound (2.86 μg lectin per mg nanoparticles). This fact may be explained by the presence of 'active' aldehyde groups. In fact, during the cross-linking process, glutaraldehyde is used to harden nanoparticles. Therefore, some unreacted aldehyde functions may remain free and react with the lectin, forming a covalent bound with lysine groups.

3.3. *In vitro activity and specificity studies*

Under the experimental conditions described above (neutral pH, 3 h of incubation time between carbodiimide and nanoparticles, and carbodiimide reagent concentration of 0.7 mg/mg nanoparticles), the UE lectin-vicilin nanoparticles conjugates (UE-VNP) typically showed an average particle size of 728 ± 15 nm (polydispersity index = 0.081), and the amount of bound lectin was calculated to be about 32 μg UE lectin/mg vicilin nanoparticle. This last value represented a UE lectin coupling efficiency of about 21% of initial ligand bulk concentration.

These UE lectin-vicilin nanoparticle conjugates were put into contact with bovine submaxillary gland mucin (BSM) which was chosen as a bio-

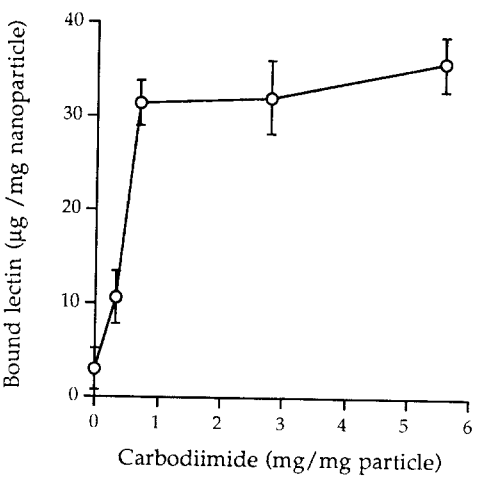

Fig. 3. Effect of increasing carbodiimide concentration on the activation step in the binding of lectin to vicilin nanoparticles. Experimental conditions: room temperature, reaction time = 3 h, pH 7.4 (PBS), $n = 3$–4.

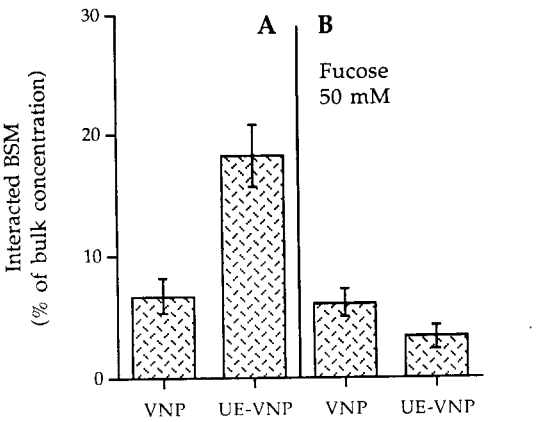

Fig. 4. BSM binding to UE lectin-vicilin nanoparticle conjugates (UE-VNP) and vicilin nanoparticles (VNP) in suspensions without (a) or with (b) 50 mM fucose. Experimental conditions: room temperature, incubation time = 60 min, pH 7.4 (PBS), bound lectin = 15 μg/ml incubation medium, VNP concentration = 1 mg/ml incubation medium, $n = 6$.

logical model to determine the in vitro activity and specificity of the conjugates towards the sugar residues of a glycoprotein. BSM is a glycoprotein whose carbohydrate part is formed by oligosaccharide chains composed of six different sugars: *N*-acetylgalactosamine (69.2 μg/mg BSM), *N*-acetylglucosamine (168 μg/mg), galactose (15.2 μg/mg), mannose (2.07 μg/mg), fucose (9.53 μg/mg) and sialic acid (16.9 μg/mg) (Honda and Suzuki, 1984). Three main peaks were separated by HPLC and their total surface was used for quantitative analysis (data not shown).

Fig. 4 represents the amounts of BSM which interacted with the UE lectin conjugates or vicilin nanoparticles (used as control) at pH 7.4, and after an incubation time of 60 min. Experiments were carried out in the absence (for the study of the in vitro activity) or in the presence of the specific sugar for UE lectin (for the study of the in vitro specificity). In the absence of L-fucose (specific sugar for UE lectin), UE lectin-vicilin nanoparticles showed three times more interaction with BSM than the unmodified vicilin nanoparticles used as control (Fig. 4(a)). These results clearly showed that the lectin remained active after its covalent coupling to vicilin nanoparticles.

Finally, Fig. 4(b) illustrates experiments performed in the presence of fucose. On the one hand, vicilin nanoparticles (controls) gave similar results ($P < 0.05$) in the presence or in the absence of the specific sugar for UE lectin. On the other hand, the interactions between conjugates and mucin decreased strongly when the competing sugar was added. Therefore, these results clearly suggested that UE lectin-vicilin nanoparticle conjugates kept the same sugar specificity as the lectin.

4. Conclusions

Conjugates formed by the covalent linkage of UE lectin to vicilin nanoparticles can be successfully prepared by the two-stage carbodiimide method. The characterization of these systems showed that the lectin coupling efficiency was close to 20%. Moreover, the in vitro activity studies showed that these conjugates were able to bind BSM (used as a biological model) in a very efficient way. Finally, UE lectin-vicilin nanoparticles kept the lectin specificity after coupling, because the presence of L-fucose strongly inhibited their interaction with mucin.

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