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Covalent coupling of asparagus pea and tomato lectins to poly(lactide) microspheres

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Abstract

Lectin–poly(lactide) microsphere conjugates specifically designed for oral administration were prepared and their activity and specificity in presence of mucus were characterized. The presence of hydroxyl or amino groups suitable for covalent coupling of lectins by the glutaraldehyde method at the surface of the microspheres have been ensured by preparing the particles in presence either of poly(vinyl alcohol) (PVA) or bovine serum albumin (BSA). Tomato and asparagus pea lectins could be covalently attached to these particles (1.0–1.3 mg/m^2 of particles). The conjugates demonstrated a 4–10 fold increase in their interactions with mucus compared to control particles. Moreover, the sugar specificity of the lectins was maintained.

Keywords: Tomato lectin; Asparagus pea lectin; Microparticles; Conjugates

1. Introduction

Biodegradable micro- and nanoparticles are promising delivery systems for improving the oral delivery of poorly absorbed drugs, including peptides, proteins or vaccines (Maincent et al., 1986; Damgé et al., 1988, 1990; Bernkop Schnürch et al., 1997; Mathiowitz et al., 1997). It has been suggested that bioavailability enhancements could mainly result from a decrease in the transit time compared to larger dosage forms and an increase in the local concentration gradient across the absorptive membrane due to bioadhesion phenomena (Ponchel and Irache, 1998). Additionally, particle uptake mechanisms have been described which take place after contact with the mucosal surface (Florence, 1997) and can contribute to absorption enhancements.

Bioadhesive interactions with mucosal surfaces can be built either on non-specific interactions depending on the physico-chemical properties of the polymer (Durrer et al., 1994a,b) or on the development of specific interactions. Specifically adhesive systems have been prepared by conjugating adhesion ligands either to polymers (Bernkop Schnürch et al., 1997) or to particulates, including nanoparticles (Irache et al., 1994b; Ezpeleta et al., 1996; Hussain et al., 1997; Hussain and Florence,

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1998; Ezpeleta et al., 1999) and liposomes (Uwiera et al., 1992; Chen et al., 1996). Among a series of recognition molecules, lectins are potentially promising ligands. Lectins are proteins which bind specifically to sugars, and therefore agglutinate cells and polysaccharides (Goldstein et al., 1980). Their conjugation to polymeric nanoparticles has been shown to be efficient for increasing the interactions with mucus (Irache et al., 1994a, 1996) and/or the surface of epithelial cells (Lehr et al., 1992) and lectins have been shown to promote particle translocation (Irache et al., 1994b; Lehr and Pusztai, 1995; Russell-Jones et al., 1999). Additionally, lectins can probably be useful for targeting specific areas of the gastrointestinal tract, due to the heterogenous distributions of sugars moieties along the normal or diseased intestine (Ponchel and Irache, 1998).

The association of lectins to particulate systems can be achieved by different procedures, including covalent linkage and adsorption processes. The conjugation should not affect the activity and specificity of the lectin. Ideally, the ligand should be bound to particles through a covalent linkage, which is more stable than simple adsorption.

Data regarding the preparation of lectin-particle conjugates are presently rather scarce. On the one hand, most conjugates have been prepared from amino- or carboxylated polystyrene nanoparticles (Naisbett and Woodley, 1990; Lehr et al., 1992; Irache et al., 1994b; Lehr and Pusztai, 1995; Haltner et al., 1996) or liposomes (Uwiera et al., 1992; Chen et al., 1996), using glutaraldehyde or carbodiimide as coupling agents. On the other hand, there are only few studies concerning biodegradable particles (Ezpeleta et al., 1996, 1999). However, non-degradable polystyrene particles were generally used as model particles, although oral administration should require the use of biodegradable polymers since these particles are likely to be, at least partially, intestinally absorbed and/or translocated by epithelial cells. Therefore, there is an interest in the development of suitable techniques for the grafting of lectins on the surface of biodegrable polymeric particles.

The tomato lectin (TL) and asparagus pea lectin (AL), specific for oligomers of *N*-acetyl-D-glucosamine and L-fucose, respectively, have been used in this study. The TL presents ex vivo a notable binding to the intestinal mucosa (Naisbett and Woodley, 1994) and has been described to be non-toxic and resistant to digestion in the gastrointestinal tract (Kilpatrick et al., 1985). Concerning the AL, a distribution of the binding sites all along the epithelium has been shown on the rat intestine (Etzler and Branstrador, 1974).

In this context, the aim of this work was to prepare by covalent coupling lectin–poly(lactide) microsphere conjugates, specifically designed for oral administration and to characterize their activity and specificity in presence of mucus.

2. Materials and methods

2.1. Materials

Poly(D,L-lactide) (PLA) Resomer® 206 was supplied by Boehringer-Ingelheim (Germany). Poly(vinyl alcohol) (PVA) (Mowiol 4–88, mw: 31 000–88% mol hydrolysis) was a gift from Hoechst (Paris, France). Bovine serum albumin (BSA, mw: 69 000), Asparagus pea lectin (mw: 54 000 (Irache et al., 1994b)), Tomato lectin (mw: 75 000 (Irache et al., 1994b)), crude gastric pig mucin and glutaraldehyde (25% aqueous solution) were purchased from Sigma (St Quentin-Fallavier, France). $Na^{125}I$ was purchased from Amersham (France). Ultrapure water was obtained from a Millipore purification system (milli-Q plus, Millipore, St Quentin en Yvelines, France). Other chemicals were analytical grade.

2.2. Preparation of PLA microspheres coated with PVA or BSA

PLA microspheres were prepared by emulsification-solvent evaporation. Either PVA or BSA were used as stabilizers and PVA/PLA microspheres or BSA/PLA microspheres were obtained, respectively.

2.2.1. Preparation of PVA/PLA microspheres

One gram of PLA was dissolved in 10 ml of methylene chloride. This solution was emulsified in 100 ml of an aqueous solution of PVA (5%

w/v) with an Ultra-turrax T25 (Janke and Kundel IKA Labortechnic) at 24 000 rpm, 5 min. To allow evaporation of solvent, emulsion was stirred overnight at room temperature with a propeller stirrer at 1400 rpm.

2.2.2. Preparation of BSA/PLA microspheres

Two hundred and sixty milligrams of PLA were dissolved in 5 ml of methylene chloride and emulsified in 80 ml of an aqueous solution of BSA (25% w/v) using an Ultra-turrax (24 000 rpm, 3 min). The emulsion was stirred overnight at room temperature with a magnetic stirrer.

In both cases, solid microspheres were collected by centrifugation ($3300 \times g$, 15 min) and washed six times with milli-Q water. The suspensions were stored at 4°C. The particle concentration was determined by weighing 1.5 ml of suspension after freeze-drying.

2.3. Microspheres characterization

The size distribution and the mean diameter of the different preparations were determined using a Coulter counter Multisizer II (Coultronics, Margency, France) fitted with a 50 μm orifice tube.

2.4. PVA determination

The amount of PVA associated to the microspheres was determined according to a colorimetric method proposed by Joshi et al. (1979) and based on the formation of a coloured complexe between two adjacent hydroxyl groups of PVA and an iodine molecule I_2. Briefly, 5 mg of six times washed and lyophilized microspheres were degraded in 2 ml of a 0.5 N sodium hydroxide solution for 15 min at 60°C. The solution was neutralized with 900 μl of 1N HCl and completed to 5 ml with milli-Q water. Three milliliter of a 0.65 M solution of boric acid (H_3BO_3), 0.5 ml of a solution of I_2/KI (0.05M/0.15M) and 1.5 ml of milli-Q water were added to the neutralized solution. Finally, the absorbance of the solution was determined at 690 nm after a 15 min incubation (Spectrophotometer Lamda 11-Perkin Elmer).

2.5. BSA determination

The amount of BSA associated to the microspheres was determined by the Lowry method. Briefly, 2.5 mg of six times washed microspheres were incubated in 1 ml of a 0.5 N sodium hydroxide solution for 3 h. The BSA content was determined by a colorimetric method, using a commercially available kit (Bio-Rad protein assay, Ivry sur Seine, France).

2.6. Preparation of lectin–microsphere conjugates

Lectins were covalently conjugated to the PVA or BSA anchored to the surface of the microspheres by using glutaraldehyde. A two step procedure, including an activation step and a coupling step was adopted. Microspheres were firstly activated, and further incubated with lectins.

PVA/PLA microspheres were activated as follows. Fifty milligrams of particles were washed in milli-Q water by centrifugation ($3300 \times g$, 10 min). The pellet was resuspended by vortexing in 750 μl of milli-Q water. Further, 1 ml of glutaraldehyde and 250 μl of H_2SO_4 0.3 M were added. The mixture was then shaken gently for 1 h at 30°C to activate the hydroxyl groups, a longer time leading to aggregation.

BSA/PLA microspheres were activated as follows. Fifty milligrams of particles was washed twice in phosphate buffer saline (PBS 10 mM, pH 7.4) by centrifugation ($3300 \times g$, 10 min). The pellet was resuspended by vortexing in 1 ml of PBS. Further, 1 ml of glutaraldehyde was added. The mixture was shaken gently for 6 h to activate the amino groups.

The activated PVA/PLA or BSA/PLA microspheres were conjugated to the lectins. Briefly, the suspension was centrifuged to remove unreacted glutaraldehyde and further washed four times in phosphate buffer saline (PBS 10 mM, pH 7.4) to remove any remaining traces of glutaraldehyde which might otherwise cross-link the lectin molecules. Then, 900 μl of PBS containing 250 μg of lectins were added and the linkage was made by incubation overnight at room temperature. The conjugates were centrifuged to remove free

lectins and incubated 1 h with 1 ml ethanolamine (0.1 M) to block unreacted groups on the particles. The ethanolamine was eliminated and the microspheres were washed three times by centrifugation. The lectin–microphere conjugates were finally resuspended in 1 ml of PBS and stored at 4°C.

2.7. Determination of the amount of bound lectins

The amount of lectin attached to the microspheres was calculated as the difference between the lectin added initially and the lectin recovered in solution after incubation with the particles. Briefly, the amount of lectin was quantified by the colorimetric determination of the amount of proteins in the supernatant (Bio-Rad protein assay).

2.8. In vitro BSA release

Previously washed ($3300 \times g$, 10 min, four washings) activated BSA/PLA microspheres (50 mg) were placed either in phosphate buffered saline (PBS 10 mM, pH 7.4) or in phosphate buffered saline containing 2% of sodium dodecylsulfate. Aliquots were collected at specific times and centrifuged ($3300 \times g$, 10 min) and BSA in the supernatant was determined using the Lowry assay (Bio-Rad protein assay). Non-activated microspheres were used as a control.

2.9. In vitro activity and specificity of the conjugates

Pig gastric mucin was used for determining in vitro the activity of the lectins after conjugation to the microspheres. Mucin was radiolabelled with ^{125}I by the chloramine T method. A solution of mucin (1 mg/ml in PBS 10 mM, pH 7.4) was prepared and filtered (Low proteic adsorption filters, porosity 0.45 μm, Millipore, St Quentin en Yvelines, France) before use. 100 μl of this solution were incubated in a solution of ^{125}INa in presence of 20 μg of chloramine T for 1 min. Further, radiolabelled mucin was separated from the excess of free iodine on a G10 sephadex column.

Thirty microliters of ^{125}I mucin were incubated for 60 min with an aliquot of the conjugates corresponding to 40 μg of bound lectins. Free ^{125}I mucin was eliminated by centrifugating the microspheres three times ($7950 \times g$, 4 min). Finally, the radioactivity of the microspheres was determined with a gamma counter.

The preservation of the activity of the lectins after coupling was determined by incubating the conjugates in ^{125}I mucin and in presence of the lectin specific sugars. α-L-Fucose (200 μl, 50 mM) and a saturated solution of chitin (a polymer of *N*-acetyl-D-glucosamine) were used for blocking the asparagus pea (AL) and tomato (TL) lectins, respectively. Non-conjugated microspheres were used as controls.

3. Results

3.1. Characterization of the microspheres

The preparations were characterized by narrow size distributions. Their mean diameters were in the micron-range and depended on the nature of the stabilizer (Table 1).

After six washings, 38.3 and 3.51 mg/m^2 (10.0 and 1.3% w/w) of BSA and PVA, respectively, remained firmly associated to the microspheres (Table 1). It was likely that at least a fraction of

Table 1
Characteristics of PLA microspheres before coupling procedures ($n = 2$)

Microspheres	Mean diameter (μm)	10% undersized (μm)	90% undersized (μm)	Stabilizer content after six washings (%w/w)
BSA/PSA	2.6	1.2	5.2	10.1
PVA/PLA	1.6	1.1	2.0	1.3

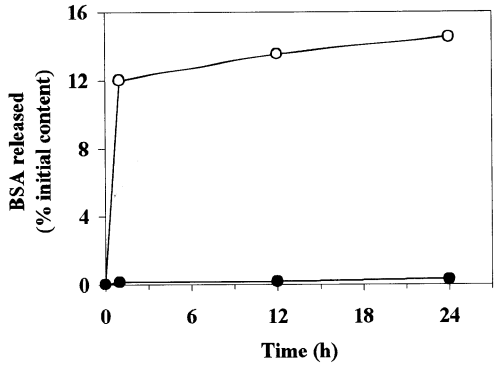

Fig. 1. Release of BSA from non-activated (○) and glutaraldehyde activated (●) PLA microspheres in a 2% v/v sodium dodecylsulfate (SDS) solution in phosphate buffer (PBS/SDS 2%, pH 7.4) ($n = 3$).

these polymers was close to the surface of the microspheres. However, as depicted in Fig. 1 for BSA, it was necessary to stabilize the peripheral layer to avoid further leaching from the particles.

Therefore, the anchorage of these polymers to the PLA core of the particles was reinforced by glutaraldehyde cross-linking. The release of BSA was dramatically lowered after glutaraldehyde activation, even when surfactant-containing phosphate buffered saline was used (less than 0.3% released after 24 h in SDS containing PBS, compared to 14% before cross-linking), suggesting that crosslinking of BSA occurred at least at the surface of the particles.

3.2. Lectin conjugates

Activated microspheres were used for lectins conjugation. Lectins conjugation to the microspheres depended on the nature of the stabilizer used for their preparation. In the case of BSA/PLA microspheres, maximal surface concentration of lectins was rapidly attained when increasing the concentration of glutaraldehyde (Fig. 2). On the contrary, the amount of fixed lectins was increased progressively in the case of PVA/PLA microspheres and no maximum could be reached. The differences in the cross-linking reactions of the stabilizing layer before covalent coupling of the lectin takes place may explain these trends.

The yield of the grafting procedure was comprised between 46 and 75% depending mostly on the coating agent. The surface concentration of the lectins was in the range of 1.0–1.3 mg/m^2, close to the values obtained for lectin grafting on polystyrene particles (Irache et al., 1994b). Surface concentration was not influenced by the nature of the lectin and depended only on the type of microspheres used (Table 2), the highest surface concentration being observed for BSA/PLA microspheres.

3.3. Activity and specificity of lectin conjugates

The interaction of the microspheres with pig gastric mucin was increased after conjugation to the lectins, suggesting that they retained their activity after covalent coupling (Fig. 3).

The affinity of the conjugates for pig gastric mucin was 4–10 times higher than the non-specific interactions observed for the non-conjugated particles. The activity depended simultaneously on the nature of the lectin and the microspheres. In absence of sugars and for the tomato lectin (TL), activities were 2.5 times higher for BSA/PLA conjugates compared to PVA/PLA conjugates. On the contrary, activities of the asparagus pea lectin (AL) conjugates were comparable.

When the activity of the TL- and AL- conjugates were tested in presence of specific sugars (chitin, a polymer of *N*-acetyl-D-glucosamine and

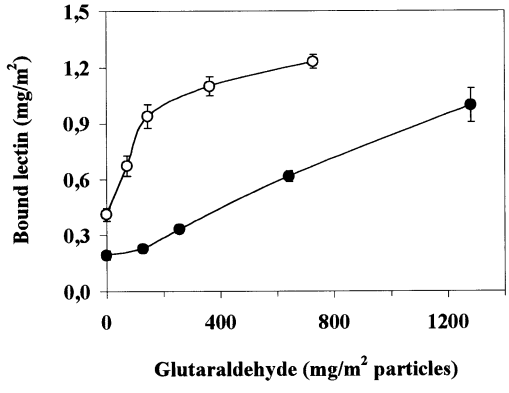

Fig. 2. Effect of the glutaraldehyde concentration on the coupling efficiency of asparagus pea lectin on PLA microspheres prepared in presence of BSA (○) or PVA (●) ($n = 3$).

Table 2
Yield of lectin grafting (expressed as '% of initial lectin amount') and surface lectin concentration, (expressed in 'mg of lectin/m^2 of particles') of PLA microspheres prepared in presence of BSA or PVA ($n = 3$)

	Yield of lectin grafting (% of initial lectin amount)		Lectin surface concentration (mg of lectin/m^2 of particles)	
	AL	TL	AL	TL
BSA/PLA	46.5 ± 1.4	53.3 ± 7.4	1.01 ± 0.19	1.35 ± 0.20
PVA/PLA	66.6 ± 6.3	75.4 ± 1.7	0.79 ± 0.19	1.08 ± 0.04

α-L-fucose, respectively), interactions of the conjugates with pig gastric mucin were competitively inhibited. However, the inhibition was not complete which could suggest that the lectins exhibited a stronger affinity for their specific sugar in the environment of the glycoprotein. Additionally, non-specific interactions originating in the proteic nature of the lectins grafted at the surface of the microspheres can not be excluded. As expected, in the presence of these sugars no inhibition was found for the control particles, suggesting that non-specific interactions with mucin were not modified (Fig. 3).

4. Discussion

Different strategies can be imagined for grafting ligands at the surface of microspheres. One of those consists in grafting the ligand on the preformed particles when sufficiently reactive groups are available. The covalent coupling of lectins on amino- or carboxylic groups beared in surface by poly(styrene) nanoparticles have been reported (Irache et al., 1994b). Covalent immobilization of hirudin on films of poly(D,L-lactide-co-glycolide) has been reported (Seifert et al., 1997). However, in the case of PLA the surface concentration of hydroxyl or carboxyl groups which may be functionalized is very low since only the end groups of the PLA chains are theoretically available. Moreover, these functions are likely to be masked at the surface of microspheres, due to the presence of surface additives. Therefore, it is necessary to increase the amount of reactive functions at the surface of the microspheres. In the present case, it was attempted to indroduce a sufficient amount of hydroxyl or amino groups at the surface by employing PVA or BSA, respectively.

The solvent-evaporation method requires the use of stabilizing agents such as surfactants or macromolecules during the emulsification step. After hardening of the particles, a small amount of these agents remain generally associated to their surface, even after repeated washing cycles. In the case of surfactants, the association of the surfactant molecules to the polymer results gener-

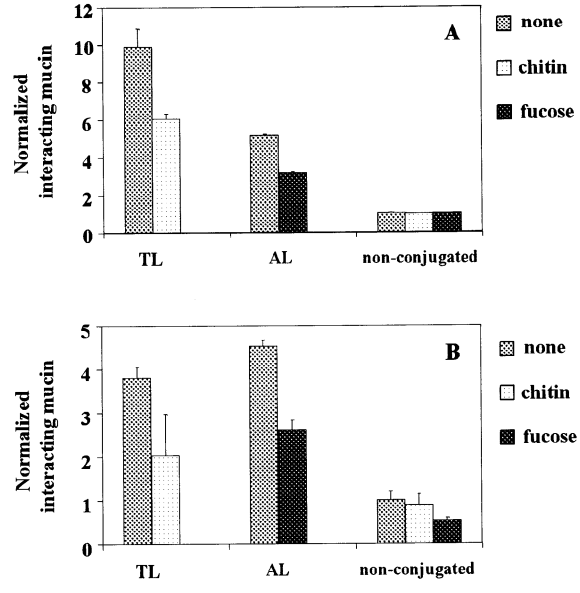

Fig. 3. Activity and specificity of PLA microspheres conjugated to tomato lectin (TL) or asparagus pea lectin (AL) prepared in presence of BSA (A) or PVA (B). The activities of the conjugates were determined by measuring the amount of mucin interacting with them in absence or in presence of specific sugars. Incubation time: 60 min. The values were normalized by reference to the non-conjugated particles BSA/PLA and PVA/PLA, respectively ($n = 3$).

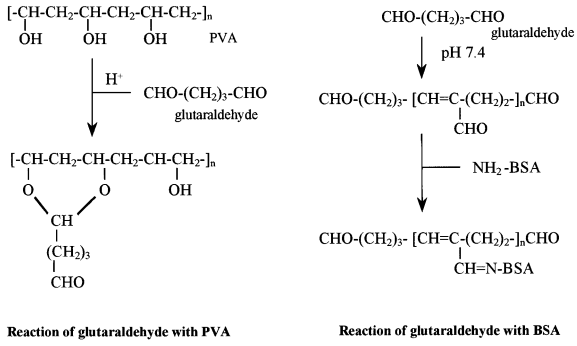

Fig. 4. Chemical reactions involved during the activation of PVA or BSA. (Monsan et al., 1975; Araujo et al., 1997).

ally from an adsorption process which may be reversible. However, in the case of macromolecules, such as PVA or BSA, a firm anchorage of the polymeric chains of PLA and PVA or BSA has been described (Boury et al., 1997) due to entanglements between polymeric chains at the surface or the sub-surface of the matrix, which result in a core-shell structure. In this respect, and not surprisingly, the amount of PVA or BSA (3.51 and 38.3 mg/m^2, respectively) associated to PLA microspheres were larger than those reported (Leonard et al., 1995) for polystyrene microspheres (2 and 0.7 mg/m^2, respectively). Additionally, in the case of BSA, covalent aggregation phenomena at the water/solvent interface has been shown to occur during the preparation of the microspheres (Crotts et al., 1997) and may lead to the high association value obtained here (10.1% w/w).

In the present study, it was shown that glutaraldehyde may help to create and to stabilize at the surface of micron-range PLA microspheres a firmly anchored layer either of BSA or PVA, which resists to detachment by surfactants. Fig. 4 describes the reactional mechanisms involved in this process. In the case of BSA, polymerized glutaraldehyde molecules, resulting at neutral or basic pH from an aldolic condensation, should react with amino groups from BSA and form an imine bond which is stabilized by the presence of a conjugated ethylenic bond (Monsan et al., 1975). In the case of PVA, the reaction of glutaraldehyde with two adjacent hydroxyl groups in acidic medium leads directly to the formation of an acetal (Araujo et al., 1997). In the two cases, free remaining aldehydic groups allow either the propagation of the cross-linking reaction or the fixation of exogeneous molecules, such as lectins.

Covalent coupling is a two step process. The moiety to be coupled should be adsorbed on the surface of the substrate before the chemical reaction between the two species can take place. In this respect, differences in adsorption during the coupling reaction can not be excluded. On the one hand, BSA/PLA microspheres from a single water-in-oil emulsion have been reported to be hydrophobic (Boury et al., 1997). On the other hand, PVA coated microspheres were probably hydrophilic and therefore, less favorable to protein adsorption (Leonard et al., 1995). The coupling efficiency depended probably partially on the reactivity and the concentration of the different species. Glutaraldehyde was very efficient in cross-linking PVA due to the high concentration in adjacent hydroxyl groups in neighbouring PVA chains. However, this was probably detrimental to the attachment of exogeneous species and may explain the slightly lower coupling efficiency observed with PVA/PLA.

The degree of activity of the lectins after coupling depend on the spatial conformation of the molecule and its ability to interact freely with substrate molecules. In this context, the flexibility and therefore the length of the attachment arm at the surface of the particles may influence the activity of the ligands. In the case of BSA and in contrast to PVA, the formation of longer and more flexible arms constituted of oligomeric chains of glutaraldehyde was likely and may probably explain the higher degree of interactions with mucin observed for BSA/PLA conjugates.

The amount of lectin immobilized on the PLA microspheres was comparable to the values obtained by Irache et al. for polystyrene nanoparticles (Irache et al., 1994b). As reported in the literature, and although it is somewhat difficult to compare these data, a large variation in the amounts of proteins grafted exists, depending on the polymer, the nature of the bifunctionnal reactive used for grafting and the protein itself. For example, Rejikumar et al. (Rejikumar and

Surekha Devi, 1995) reported pepsin coupling on PVA beads to be in the range of 8.4–9.7 mg/g of beads, which was in the range of 250 mg/m^2. Alternatively, the coupling of ovomucoid on a graft copolymer of acrylic acid and polyethylene gave only 3 mg/m^2 (Valuev et al., 1998).

5. Conclusion

This study has shown that grafting of lectins to PLA microspheres was feasible by coupling these proteins to strengthened PVA or BSA surface layers beared by the particles. Two features of lectins, i.e. binding affinity and specificity for carbohydrate moieties of mucus glycoproteins were preserved. Probably, advantage can be taken from such properties for the development of site specific mucosal drug delivery systems.

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