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International Journal of Pharmaceutics 239 (2002) 47–59

international journal of pharmaceutics

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Characterization of alginate/poly-L-lysine particles as antisense oligonucleotide carriers

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Received 14 September 2001; received in revised form 10 January 2002; accepted 23 January 2002

Abstract

The gel forming characteristics of alginate in the presence of calcium ions and further crosslinking with poly-L-lysine led to the formation of sponge-like nano- and microparticles. The particle size was varied by adjusting the final concentrations of and proportions between the components. The region for particle formation was from 0.04 to 0.08% (w/v) of alginate in the final formulation, the change from the nm to μm size range occurred at a concentration of approx. 0.055% (w/v). Oligonucleotide-loaded microparticles were prepared by two different methods, either by absorption of the drug into the crosslinked polymeric matrix or by incorporation of an oligonucleotide/poly-L-lysine complex into a calcium alginate pre-gel. The release of oligonucleotide from microparticles prepared by the first method was higher. The addition of increasing amounts of poly-L-lysine resulted in larger particles, higher oligonucleotide loading and slower drug release. An increase in the final solid content of the formulation led to larger particles, especially with high concentrated calcium alginate pre-gels. Microparticles based on alginate and poly-L-lysine are potential carriers for antisense oligonucleotides.

Keywords: Alginate; Antisense oligonucleotide; Complexation; Microparticles; Nanoparticles; Poly-L-lysine

1. Introduction

The development of new delivery systems for the administration of gene therapeutics is a field of great interest. The pharmacological basis of antisense technology is to down-regulate the gene expression of disease-causing proteins at the level of mRNA (Crooke, 1993; Akhtar et al., 2000). This principle permits the rational design of sequence-specific nucleic acid drugs, which target and inhibit a particular mRNA by Watson–Crick hydrogen bonding (Sohail and Southern, 2000).

Antisense oligonucleotides (phosphorothioate backbone chemistry) are polyanionic molecules with 10–25 nucleotides and a molecular weight from 3 to 8 kDa. They are hydrophilic ($\log D_{(\text{octanol/water})} = \text{approx. } -3.5$) and do not readily pass biological membranes (Nicklin et al., 1998; Wu-Pong, 2000). In addition, pre-systemic degradation, nuclease metabolism and non-spe-

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0378-5173/02/$ - see front matter
PII: S0378-5173(02)00030-3

cific adhesion to biological surfaces decrease the availability of oligonucleotide at the target tissues, and therefore reduce the chances of cellular uptake of therapeutic doses (Agrawal and Zhang, 1998). In order to enhance the biological stability of these compounds, chemically-modified oligonucleotides with structural changes in the phosphate backbone or in the nucleotide sugar have been developed (Cummins et al., 1995; Cook, 1998). In phosphorothioate oligonucleotides, one of the non-bridging oxygens is replaced by sulfur. This results in a higher resistance against enzymatic degradation without compromising hybridization efficiency. The optimization of oligonucleotide delivery requires improved absorption via non-parenteral routes, correct targeting (in time and space), enhanced cellular uptake and adequate intracellular trafficking (Akhtar et al., 2000). Recently, many gene vectors based on non-viral carrier systems have been described and include cationic liposomes (Kawakami et al., 2000; Lebedeva et al., 2000), lipoplexes, dendrimers (Shchepinov et al., 1999; Yoo et al., 1999) and several other polycationic polymers (Arigita et al., 1999; Kabanov, 1999; Vinogradov et al., 1999).

Alginate is an anionic polysaccharide derived from brown-algae cell-walls and consists of guluronic and mannuronic acid units linked with glycosidic bonds. Gelation can be induced by cross-linking of the guluronic acid units with di- or polyvalent cations (Bystrický et al., 1990; Aslani and Kennedy, 1996). Alginate has been reported to be mucoadhesive, biodegradable and biocompatible (Lemoine et al., 1998), and is therefore interesting for oligonucleotide delivery. Poly-L-lysine is a polycationic drug carrier known to increase the uptake efficiency of antisense oligonucleotides by non-specific adsorptive endocytosis. The application of poly-L-lysine as a drug carrier is limited due to its potential toxicity, which can be reduced by the use of lower molecular weight fractions and the coadministration with polyanions (Dokka and Rojanasakul, 2000). Microcapsules based on alginate and poly-L-lysine have been shown to successfully protect living endocrine tissues from immune rejection after transplantation (De Vos et al., 1997; Lanza et al., 1999; Leblond et al., 1999). In addition, the suitability of these systems for oral and/or nasal antigen delivery has been demonstrated in several studies (Bowersock et al., 1996; Lemoine et al., 1998; Bowersock et al., 1999). Recently, alginate-based nanoparticles were reported to protect an associated oligonucleotide from degradation in bovine serum and to modify its bio-distribution after intravenous administration (Vauthier et al., 1998; Aynie et al., 1999; Lambert et al., 2001).

The objectives of this work were to study the ionotropic gelation properties of sodium alginate in the presence of calcium ions and poly-L-lysine and to characterize the impact of the concentration and the proportion between components on the size distribution of the resulting particles. The comparisons focused on selected systems in order to reduce the costs of the study. In addition, the association and release characteristics of phosphorothioate oligonucleotides to alginate/poly-L-lysine particles were analyzed as a function of formulation and preparation method. Alginate-based microparticles loaded with oligonucleotide are potential carriers to enhance the oral bioavailability of antisense therapeutics (González Ferreiro, 2001).

2. Materials and methods

2.1. Materials

Poly-L-lysine hydrobromide (PLL) with MW 9600 and 9400 Da, alginic acid sodium salt (Na-alginate) from Macrocystis pyrifera with medium viscosity (viscosity of 2% solution at 25 °C, approx. 3500 cps), calcium chloride anhydrous or dihydrate, tris[hydroxymethyl]aminomethane (Trizma®) hydrochloride, sodium bromide, potassium chloride and acetonitrile (Sigma, St. Louis, MO) were used as received. Ultrapure Milli-Q® water was used throughout the study. Phosphate buffer pH 7.4 was prepared according to the USP XXIII.

ISIS 5132 (5′-TCC CGC CTG TGA CAT GCA TT-3′) phosphorothioate oligodeoxynucleotide (PS-ODN) inhibitor of C-raf kinase with MW 6348 Da and ISIS 2302 (5′-GCC CAA GCT GGC ATC CGT CA-3′) PS-ODN with MW 6781 Da

designed to inhibit human intercellular adhesion molecule (ICAM-1) expression, were synthesized at Isis Pharmaceuticals following the deoxynucleoside phosphoroamidite method (Bennett et al., 1996).

2.2. Microparticle preparation

Alginate/poly-L-lysine/oligonucleotide microparticles were obtained in an aqueous environment, based on the ionotropic gelation of alginate with calcium chloride followed by crosslinking with poly-L-lysine (Rajaonarivony et al., 1993). An aliquot of a 0.09% (w/v) Na-alginate solution was mixed under magnetic stirring with an 18 mM $CaCl_2$ stock solution, then deionized water was added to yield Ca-alginate pre-gels with a Na-alginate concentration of 0.06 or 0.08% (w/v) and a Ca- concentrations of 0.9 or 1.2 mM. An example formulation of the Ca-alginate pre-gel consisted of 88.9 ml of 0.09% (w/v) Na-alginate solution, 6.7 ml of 18 mM $CaCl_2$, and 4.4 ml or 37.8 ml of deionized water to produce pre-gels of 0.08 or 0.06% Na-alginate, respectively. The ratio of Na-alginate:$CaCl_2$ was maintained constant at 6:1 (w/w) in all cases for the preparation of the microparticles. Also, equivalent pre-gel systems were prepared with different final volumes to give different batch sizes. Crosslinking with poly-L-lysine was achieved by two different approaches (Vauthier et al., 1998). In the first approach (method 1), a certain volume of a 0.3, 0.06 or 0.03% (w/v) poly-L-lysine stock solution was added to the Ca-alginate pre-gel under magnetic stirring (30 min) leading to microparticle formation. The oligonucleotide was dissolved in an aliquot of the liquid phase of the dispersion in order to avoid further dilution of the system and added back to the particle dispersion, followed by equilibration for 4 days under stirring (Rajaonarivony et al., 1993). In the second approach, the oligonucleotide was added to a 0.3% (w/v) poly-L-lysine stock solution leading to the formation of a colloidal complex, which was then incorporated into the Ca-alginate pre-gel and stirred for 4 days (method 2).

The oligonucleotide association capacity of the microparticles was evaluated after separation of the microparticles from the aqueous medium by centrifugation (3500 rpm, 30 min). The amount of free oligonucleotide in the supernatant was determined in triplicate by UV-spectrophotometry at 260 nm (UV-2101 PC, Shimadzu Scientific Instruments, Columbia, MD). The oligonucleotide association capacity was calculated as the % association capacity $= 100 \times$ (total mg of ODN $-$ free mg of ODN)/(total mg of ODN).

After separation, the supernatant was eliminated and the microparticles were frozen at -70 °C, lyophilized in a Christ Alpha I-5 freeze-drier (Martin Christ Gefriertrocknungsanlagen, Osterode, Germany) and stored in a desiccator.

2.3. Determination of the ODN loading of the microparticles

The oligonucleotide loading in the dried microparticles was determined after extraction with a strong anionic exchange (SAX) buffer consisting of 0.12% (w/v) Trizma® hydrochloride, 3.7% (w/v) KCl, 10.3% (w/v) NaBr, 30% (v/v) acetonitrile and water, adjusted to pH 9. The microparticles (5–10 mg) were placed into 25 or 50 ml of SAX buffer pH 9 and agitated for 2 h in a horizontal shaker ($n = 3$) (IKA HS 501, Janke & Kunkel IKA Labortechnik, Staufen, Germany). The ODN concentrations were determined by UV-spectrophotometry and the ODN loading was calculated as % loading $= 100 \times$ (amount of extracted ODN)/(amount of dried microparticles).

2.4. Particle size analysis

The particle size distribution of the microparticles was analyzed by laser light scattering with a Horiba® LA 910 equipment (Horiba, Kyoto, Japan) or a Coulter® LS 230 equipment (Coulter Electronics, Krefeld, Germany, small volume module). The relative frequency of the diameter based on the volume distribution of the particles is presented ($n = 3$).

2.5. Scanning electron microscopy

The shape and surface characteristics of the microparticles were studied by scanning electron microscopy (SEM). The particles were coated with gold-palladium and then observed with a scanning electron microscope (Philips SEM 515, PW 6703, Philips Optical Electronics, Eindhoven, Netherlands).

2.6. Release studies

Lyophilized microparticles (approx. 10 mg) were placed into flasks containing 50 ml of release medium (Milli-Q® water or phosphate buffer pH 7.4) at 37 °C and shaken horizontally at 75 rpm ($n = 3$) (GFL 3033, Gesellschaft für Labortechnik, Burgwedel, Germany) for 24 h. A total of 2 ml samples were taken at different time intervals, replaced with fresh release medium and analyzed by UV-spectrophotometry at 260 nm.

3. Results and discussion

3.1. Gelation and crosslinking of Na-alginate: identification of the region for particle formation

To determine the upper concentration limit in the gel forming properties of alginate in the presence of calcium cations, several systems with increasing alginate concentration and total solids content were prepared. A total of 1 ml of an 18 mM $CaCl_2$ stock solution was added to Na-alginate stock solutions (9 ml, concentrations ranging from 0.18 to 0.44% w/v). Table 1 shows the final composition of each system and their corresponding macroscopic evaluation. A proportion of Na-

Table 1
Gelation of Na-alginate with $CaCl_2$: macroscopic evaluation at different total solids contents and ratios between Na-alginate and calcium

Formulation		Na-alginate/$CaCl_2$ (w/w)	Total solids content (mg/ml)	Macroscopic evaluation
% Na-alginate	mM Ca			
0.16	1.80	8:1	1.8	Homogeneous gel
0.20	1.80	10:1	2.2	Homogeneous gel
0.27	1.80	13:1	2.9	Thick gel
0.40	1.80	20:1	4.2	Gel with lumps

Table 2
Gelation of Na-alginate with $CaCl_2$ followed by crosslinking with poly-L-lysine (MW 9600 Da): effect of increasing the solids content in the formulation on the resulting particle size at a constant ratio of Na-alginate/$CaCl_2$/PLL of 6:1:1.15 (w/w/w)

Formulation			Solids content (mg/ml)	Macroscopic evaluation	Median size (μm)
% Na-alginate	mM Ca	μM PLL			
0.010	0.150	2.005	0.136	Solution	–
0.030	0.451	6.016	0.408	Solution	–
0.040	0.601	8.019	0.544	Opalescent	0.136 ($\pm$0.012)
0.050	0.751	9.983	0.679	Opalescent	0.339 ($\pm$0.011)
0.055	0.827	11.025	0.748	Opalescent	0.434 ($\pm$0.015)
0.060	0.901	12.021	0.815	Suspension	15.618 ($\pm$0.175)
0.070	1.051	14.021	0.951	Suspension	23.966 ($\pm$0.063)
0.080	1.202	16.026	1.087	Suspension	143.012 ($\pm$0.202)
0.090	1.352	18.026	1.223	Aggregates	–
0.100	1.502	19.965	1.358	Aggregates	–

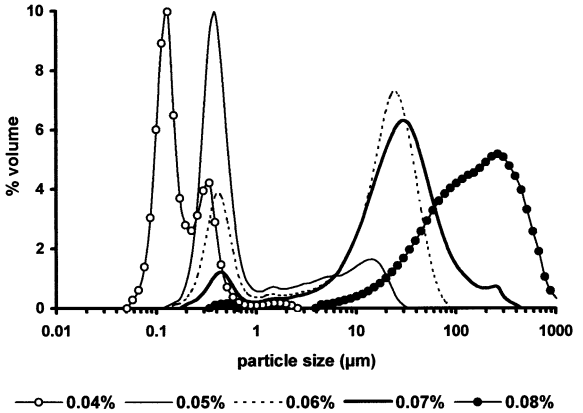

Fig. 1. Particle size analysis of different systems formulated at a constant ratio of Na-alginate/$CaCl_2$/PLL of 6:1:1.15 (w/w/w) and increasing concentrations. The symbols indicate system concentrations expressed as % of Na-alginate (w/v) in the final preparation.

alginate to $CaCl_2$ of 10:1 (w/w) was the maximum limit to obtain a homogeneous Ca-alginate pre-gel for further crosslinking with poly-L-lysine.

The calcium-induced gelation of alginate occurs mostly on the guluronic blocks of the polymer chains in an 'egg-box' fashion, yielding a pre-gel state with microdomains of concentrated alginate. Poly-L-lysine, a multivalent cationic poly-amino acid that is likely to have more affinity for the mannuronic residues (Sime, 1990), was used to further crosslink the pre-gel's microdomains into particles.

In the following experiments, poly-L-lysine was added to the Ca-alginate pre-gel in order to determine the concentration range for the formation of particles. The effect of increasing solids content on the particle size was evaluated at a fixed alginate/$CaCl_2$/PLL ratio of 6:1:1.15 (w/w/w). Particles were obtained for solids contents of 0.544–1.087 mg/ml corresponding to Na-alginate concentrations between 0.04 and 0.08% (Table 2), lower and higher concentrations led to solution-like systems or to larger aggregates. The mobility of Ca-alginate chains might be restricted as the total solids content in the system increased, therefore, tending to form larger structures when a crosslinking agent such as poly-L-lysine was incorporated. Increasing the solids content resulted in an increase in particle size, the transition from nanoparticles to microparticles occurred at a solids content of 0.815 mg/ml and a Na-alginate concentration of 0.060% (Fig. 1). The change in particle size with the alginate content followed an exponential relation $y = 5 \times 10^{-5} e^{187.82x}$ ($R^2 = 0.90$), where y was the particle size (μm) and x the % alginate (as initial Na-alginate).

Our objective was to obtain particles in the μm size range to facilitate the large-scale production, further isolation and drying of these systems to be included in solid dosage forms intended for a potential gastrointestinal application. Ca-alginate pre-gels at Na-alginate concentrations of 0.06 and 0.08% (w/v) were prepared at a fixed ratio of Na-alginate to $CaCl_2$ of 6:1 (w/w). Then, increasing volumes of 0.03, 0.06 and 0.3% (w/v) poly-L-lysine stock solutions were added to proceed with crosslinking. In the case of an initial pre-gel with

Table 3
Crosslinking of a Ca-alginate pre-gel [0.06 % (w/v) in Na-alginate], with increasing volumes of 0.03, 0.06 and 0.3% poly-L-lysine (MW 9600 Da) solutions

Na-alginate/$CaCl_2$/PLL (w/w/w)	Total solids content (mg/ml)	Macroscopic evaluation	Median size (μm)
6:1:0.3	0.664	Solution	–
6:1:0.6	0.633	Light opalescent	0.384 ($\pm$0.007)
6:1:0.9	0.608	Light opalescent	0.494 ($\pm$0.015)
6:1:0.6	0.691	Light opalescent	0.514 ($\pm$0.019)
6:1:1.2	0.683	Opalescent	4.527 ($\pm$0.022)
6:1:1.8	0.677	Opalescent	129.978 ($\pm$0.034)
6:1:3.0	0.909	Suspension	240.374 ($\pm$0.117)
6:1:6.0	1.083	Suspension	269.646 ($\pm$0.056)
6:1:9.0	1.231	Suspension	256.961 ($\pm$0.272)

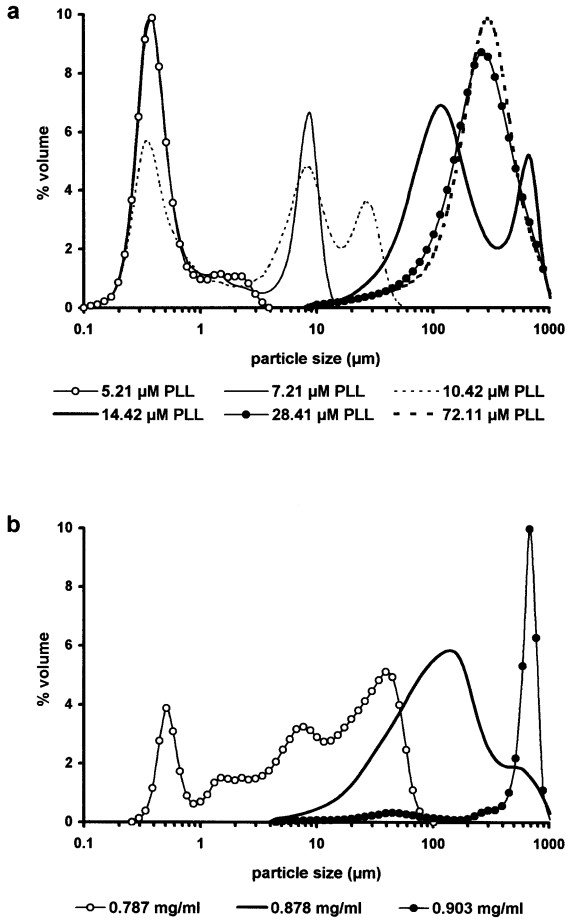

Fig. 2. Effect of the Ca-alginate pre-gel concentration, incorporated amount of poly-L-lysine (9600 MW) and total solids content on the resulting particle size. (a) Effect of increasing poly-L-lysine (PLL) concentration on the particle size of systems formulated from a Ca-alginate pre-gel with a 0.06% (w/v) Na-alginate content. (b) Effect of final total solids content (mg/ml) on the particle size of systems formulated from a Ca-alginate pre-gel containing 0.08% (w/v) of Na-alginate.

0.06% Na-alginate, the effect of incorporating higher amounts of poly-L-lysine had a larger influence on the resulting particle size than the effect of total solids in the system (Table 3). Systems with a constant ratio of alginate/$CaCl_2$/PLL of 6:1:0.6 (w/w/w) and different total solids content, 0.633 and 0.691 mg/ml, showed little variation in the particle size (384 and 514 nm, respectively). On the other hand, systems with similar total solids content such as 0.664 and 0.677 mg/ml but increasing amount of poly-L-lysine (ratio of alginate/$CaCl_2$/PLL (w/w/w) of 6:1:0.3 and 6:1:1.8, respectively) markedly switched from a solution to an opalescent system with microparticles of 130 μm. Particles had a size in the nanometer range at lower poly-L-lysine concentrations and had a larger size at a concentration around 10 μM and higher (Fig. 2a).

Systems from a pre-gel with a higher concentration of Na-alginate (0.08%) were prepared in the same fashion. Both the increase in poly-L-lysine and the total solids content in the final formulation controlled the increase in particle size. Particles in the micrometer range were already obtained at a poly-L-lysine concentration around 5 μM. In this case, the systems with a constant ratio of alginate/$CaCl_2$/PLL of 6:1:0.45 (w/w/w) and different total solids content, 0.828 and 0.903 mg/ml, showed a great difference in the resulting particle size (10 and 605 μm, respectively) (Table 4). At higher initial amounts of alginate, a small change in the total solids content had a large effect on the particle size (Fig. 2b).

The concentration of the crosslinking agent mostly determined the final particle size for systems formulated from a diluted Ca-alginate pre-gel. The range of obtained particle sizes was widened by adapting the concentration and volume of this agent. Alternatively, for systems with higher initial Na-alginate proportions, a slight change in the concentration during formulation drastically affected the resulting particle size. Therefore, it was more difficult to regulate the obtained particle size during processing.

3.2. Effect of the order of addition on the formation of oligonucleotide-loaded and unloaded microparticles

The influence of the order of incorporating the different ingredients into the final system was investigated by particle size analysis. The goal was to elucidate the interactions established between the components during particle formation. The standard formulation consisted of 0.05% (w/v) in Na-alginate, 0.75 mM Ca, 52.08 μM poly-L-lysine (9600 Da) and 78.77 μM ISIS 5132. The particle size of the final systems was characterized after 1.5 h, 2 and 4 days (Table 5 and Fig. 3a and b).

The first particulate system was prepared following the method 1. The Ca-alginate pre-gel was treated under stirring with a 0.3% (w/v) poly-L-lysine solution leading to microparticle formation followed by drug addition after 30 min. During the first 2 days, the particle size decreased from an initial value of 185 to 27 μm and then remained almost constant up to 4 days, displaying a final size of 30 μm.

The second system was formulated according to method 2, the poly-L-lysine and the oligonucleotide were complexed first leading to a colloidal system which was then added to the Ca-alginate pre-gel. The particle size remained practically unchanged for the first 2 days with a value around 115 μm and then decreased to 57 μm after 4 days. The complexation between the oligonucleotide and poly-L-lysine without the incorporation into the Ca-alginate pre-gel led to a colloidal system with a small particle size and little variation during the 4 days of association (final size of 384 nm). The absence of alginate resulted in fine complexes with a particle size in the nanometer range, as generally reported in the complexation of antisense oligonucleotides with polycationic substances (González Ferreiro et al., 1999).

The differences in the time-dependent size changes for the investigated systems could be attributed to a different rearrangement of the polymer chains during gelation and crosslinking according to the sequence at which the substances were incorporated. When the Ca-alginate pre-gel contacted the poly-L-lysine first, the crosslinking of the polymer matrix occurred faster than if a complex of the crosslinking agent with the oligonucleotide was incorporated into the pre-gel. In the first case (preparation method 1), the availability of the positive charges of the poly-L-

Table 4
Crosslinking of a Ca-alginate pre-gel [0.08 % (w/v) in Na-alginate], with increasing volumes of 0.03, 0.06 and 0.3% poly-L-lysine (MW 9600 Da) solutions

Na-alginate/$CaCl_2$/PLL (w/w/w)	Total solids content (mg/ml)	Macroscopic evaluation	Median size (μm)
6:1:0.23	0.876	Opalescent	0.789 ($\pm$0.024)
6:1:0.45	0.828	Opalescent	10.349 ($\pm$0.085)
6:1:0.67	0.787	Opalescent	22.262 ($\pm$0.122)
6:1:0.45	0.903	Suspension	604.592 ($\pm$0.345)
6:1:0.90	0.878	Suspension	103.750 ($\pm$0.296)
6:1:1.35	0.856	Aggregates	–
6:1:2.25	1.121	Aggregates	–
6:1:4.50	1.278	Aggregates	–
6:1:6.75	1.410	Aggregates	–

Table 5
Interactions between 0.05% Na-alginate/0.75 mM Ca/52.08 μM PLL/78.77 μM ODN: effect of the order of addition and presence/absence of ingredients on the resulting particle size as a function of stirring time

System	Size (μm)			% association capacity
	1.5 h	2 days	4 days	
(PG+PLL)+ODN	184.6	27.0	30.0	95.2
(PLL+ODN)+PG	117.0	115.2	57.4	94.0
(PG+ODN)+PLL	99.2	126.5	42.0	94.1
PG+PLL	291.3	288.8	130.9	–
PLL+ODN	0.4	0.4	0.4	–
PG+ODN	–	–	–	–

PG, pre-gel; PLL, poly-L-lysine; ODN, oligodeoxynucleotide.

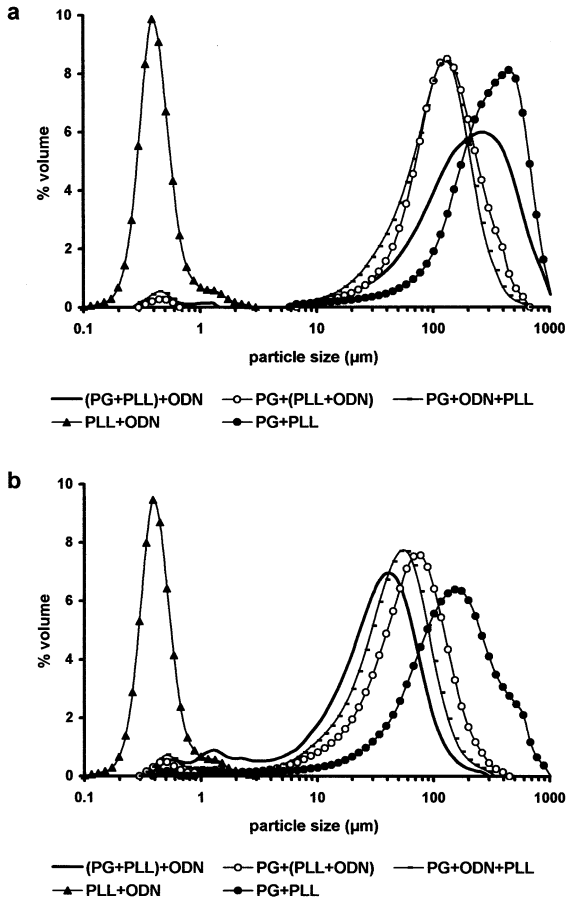

Fig. 3. Effect of the order of addition and presence or absence of ingredients on the particle size of systems based on Na-alginate, $CaCl_2$, poly-L-lysine (MW 9600 Da) and ISIS 5132 mixtures (a) after 1.5 h and (b) after 4 days.

lysine to interact with the alginate network was higher because the oligonucleotide was added afterwards. In the second case (preparation method 2), some of the positive groups of the poly-L-lysine were already bound to the negative sites of the oligonucleotide, therefore further interaction with the alginate network occurred in a slower fashion.

A third system composed of the standard formulation mixed in another sequence was investigated. The oligonucleotide was incorporated into the Ca-alginate pre-gel without apparent macroscopical change, followed by the addition of the poly-L-lysine solution, which resulted in microparticle formation. The size slightly increased during the first 2 days from an initial value of 99 to 126 μm, and then decreased to a final size of 42 μm in the second half of the study. When the oligonucleotide and the pre-gel were mixed before the addition of poly-L-lysine, the interaction between charges upon the incorporation of the crosslinker probably happened in a more competitive way and thus lacking a regular arrangement. This resulted in an increase of the particle size during early association times followed by a decrease in the size with further equilibration.

The systems consisting of the complete standard formulation displayed a similar and high association capacity independent from the order of addition of the components. About 95% of the initial oligonucleotide was incorporated into the alginate/poly-L-lysine network.

The addition of oligonucleotide at any step of the preparation method resulted in particle shrinkage. Unloaded microparticles (approx. 130 μm), formed by the addition of a concentrated poly-L-lysine solution to the Ca-alginate pre-gel, were larger than loaded microparticles.

3.3. Effect of poly-L-lysine/oligonucleotide ratio and preparation method on the association kinetics, particle size and drug release of loaded microparticles

Microparticles were formulated following the two standard methods with modified preparation sequence, and at different amounts of poly-L-lysine. Table 6 summarizes the information about the preparation method, formulation and characterization of these systems. Method 1 led to larger particles than method 2 with both investigated formulations. The analysis of the microparticles by scanning electron micrography supported this tendency. The particles formed by addition of poly-L-lysine to the Ca-alginate pre-gel followed by loading with the oligonucleotide had a larger size, probably because the alginate mass trapped into the structure was higher (Fig. 4a and c). A higher poly-L-lysine content resulted in larger particle sizes. The amount of alginate held into the particulate system may be higher with increasing amounts of crosslinking agent in the formulation, therefore resulting in larger particles.

Table 6
Characterization of microparticles produced by two methods and presenting different poly-L-lysine (PLL, MW 9400 Da) composition

Method	Formulation				Alginate/PLL/ODN (w/w/w)	Ratio PLL/ODN (+/−)	Association (%)	Loading (%)	Size (μm)
	% Na-alginate	mM Ca	μM PLL	μM ODN					
1	0.05	0.75	53.20	72.80	1:1:1	1.73	95.09	34.97	50.73 (±0.28)
2	0.05	0.75	53.20	72.80	1:1:1	1.73	98.69	39.24	29.91 (±0.55)
1	0.05	0.75	43.37	72.80	1:0.8:1	1.41	68.43	33.18	38.44 (±0.25)
2	0.05	0.75	43.37	72.80	1:0.8:1	1.41	93.87	49.45	14.79 (±0.43)

All systems were formulated with the antisense oligodeoxynucleotide ISIS 2302 (ODN). Method 1, crosslinking of the Ca-alginate pre-gel with PLL leading to microparticles then loaded with ODN. Method 2, addition of the PLL/ODN complex into the Ca-alginate pre-gel forming microparticles.

The association capacity was strongly influenced by the ratio of poly-L-lysine to the other excipients and the method of preparation. Higher association values were found for higher poly-L-lysine contents and when the complex was incorporated into the Ca-alginate pre-gel (method 2). The loading of microparticles produced by method 2 was also higher. The amount of alginate incorporated into the particles was probably lower because the poly-L-lysine first formed a complex with the oligonucleotide. This behavior was more pronounced in the formulation containing less poly-L-lysine.

The morphology of the microparticles was studied by SEM. All microparticles had a sponge-like appearance and were of irregular shape. The systems formulated with a higher amount of crosslinking agent (Fig. 4a and b) showed a surface characterized by the presence of globular structures, formed by the interaction of alginate and oligonucleotide with poly-L-lysine. A lower amount of poly-L-lysine in the formulation led to an irregular surface, probably formed by clustered poly-L-lysine and oligonucleotide complexes and just little alginate (Fig. 4c and d).

The release profiles of free oligonucleotide from freeze-dried microparticles were determined in distilled water (Fig. 5a) and phosphate buffer pH 7.4 (Fig. 5b) over a 24 h period. The amount of oligonucleotide released in water was, in general, higher than in phosphate buffer. The explanation for this behavior might be that in a deionized environment the polymer matrix swelled allowing the dissociation of the oligonucleotide. In contrast, in phosphate buffer the ionic composition of the surrounding medium caused the sponge-like structure to collapse, inhibiting somehow further release of the components. Microparticles were polyionic systems consisting of an anionic drug loaded within a polycationic–polyanionic polymer matrix. Due to the rich ionic content of the particulate systems it is likely that in environments with higher ionic strengths the polymeric structure gets packed into a more compact form.

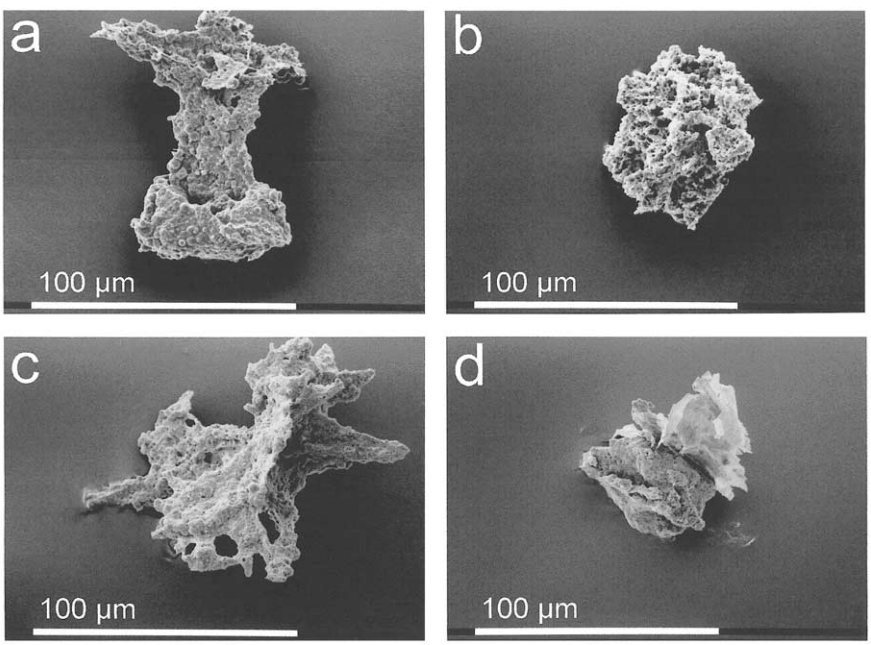

Fig. 4. Scanning electron microscopy (magnification of 6.25 E2, scale 100 μm) of freeze-dried microparticles of alginate/poly-L-lysine (9400 MW)/ISIS 2302 prepared by two different methods and with two proportions of poly-L-lysine: (a) method 1, PLL/ODN ratio of 1.73 +/−; (b) method 2, PLL/ODN ratio of 1.73 +/−; (c) method 1, PLL/ODN ratio of 1.41 +/−; and (d) method 2, PLL/ODN ratio of 1.41 +/−.

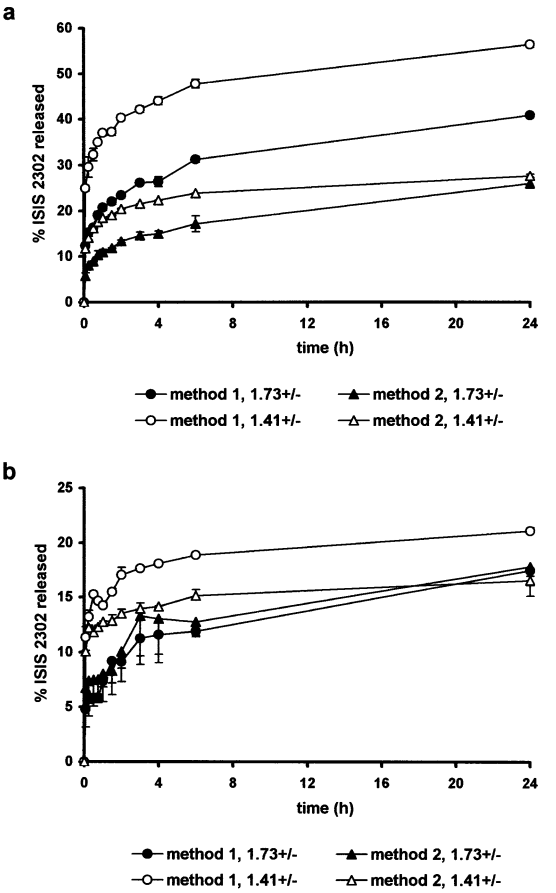

Fig. 5. Release studies, (a) in distilled water (b) in phosphate buffer pH 7.4, from freeze-dried microparticles of alginate/poly-L-lysine (9400 MW)/ISIS 2302 prepared by method 1 or method 2 and displaying a PLL/ODN ratio of 1.73 +/− or 1.41 +/−.

However, it was macroscopically observed that in phosphate buffer the formulations with less amount of poly-L-lysine (ratio PLL/ODN of 1.41 +/−) formed colloidal suspensions, probably constituted by freed complexes of poly-L-lysine with oligonucleotide. The complexed oligonucleotide could not be measured spectroscopically but might be biologically active. In other studies, we demonstrated the formation of complexes between oligonucleotide and poly-L-lysine in the nanometer range (González Ferreiro et al., 2001), thus they could potentially be taken up by the intestinal mucosa (Desai et al., 1996).

In distilled water (Fig. 5a), the impact of the preparation method on the release characteristics was clear. The formulations prepared by method 1 showed a larger extent of release, probably due to the fact that oligonucleotide was loaded into the polymeric network by aqueous diffusion after microparticle formation, thus the electrostatic interaction with poly-L-lysine was less intense. In addition, most of the oligonucleotide may have reacted with the available positive groups of poly-L-lysine at the surface of the polymeric matrix, barely getting into inner layers. Also, the systems formulated with a lower amount of poly-L-lysine released more oligonucleotide in free form than equivalently prepared ones with a higher poly-L-lysine content.

The release profiles in phosphate buffer pH 7.4 were more dependent on the amount of poly-L-lysine in the formulation rather that on the preparation method (Fig. 5b). The ratio of crosslinking agent to the other ingredients in the formulation, rather than the order of addition of the components during preparation, mostly determined the characteristics of the formed particles. Presumably, the sequence of addition had only a relative importance because the interaction for microparticle formation was mainly electrostatic and thus, ruled by the ionic composition of the formulation and the release medium.

4. Conclusions

The versatility of the ionotropic gelation of alginate with calcium ions and poly-L-lysine offered the possibility of preparing nano- and microparticles, employing mild conditions in an aqueous environment, with a wide range of sizes. The association capacity and loading of oligonucleotide by aqueous diffusion onto the sponge-like particles resulted to be very high. It was possible to obtain different in-vitro release profiles according to the poly-L-lysine/oligonucleotide ratio, preparation method and release medium. These systems are promising carriers for the administration of antisense oligonucleotides by both parenteral (Vauthier et al., 1998; Aynie et al., 1999) and non-parenteral routes (Bowersock et al., 1996; Lemoine et al., 1998).

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