Alginate/Poly-L-Lysine Microparticles for the Intestinal Delivery of Antisense Oligonucleotides

María González Ferreiro[1], Lloyd G.Tillman[2], Gregory Hardee,[2] and Roland Bodmeier[1,3]

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Purpose. A microparticle carrier based on alginate and poly-L-lysine was developed and evaluated for the delivery of antisense oligonucleotides at the intestinal site. Formulations of oligonucleotide-loaded microparticles having differences in the carrier molecular weight and composition were characterized *in vitro* and *in vivo.*

Methods. Polymeric microparticles were prepared by ionotropic gelation and crosslinking of alginate with calcium ions and poly-L-lysine. The loading of the antisense oligonucleotide into the microparticles was achieved by absorption in aqueous medium. The association capacity, loading and particle size of the microparticles were characterized. The *in vivo* performances of various formulations after intrajejunal administration were studied in rat and in dog models.

Results. Microparticles had a sponge-like structure and an oligonucleotide loading of 27–35%. The composition of the medium affected the particle size and the *in vitro* release profiles. The oligonucleotide bioavailability after intrajejunal administration to rats in the presence of permeation enhancers was good for most of the tested systems. The application of microparticles in powder form compared to an equivalent suspension improved the intrajejunal bioavailability of the oligonucleotide (25% and 10% respectively) in rats. On the contrary, the intrajejunal administration to dogs resulted in poor oligonucleotide bioavailability (0.42%).

Conclusions. The formulation of antisense oligonucleotides within alginate and poly-L-lysine microparticles is a promising strategy for the oral application.

KEY WORDS: antisense oligonucleotide; alginate; poly-L-lysine; permeation enhancer; microparticles; intestinal absorption.

INTRODUCTION

Antisense oligonucleotides are a novel class of therapeutic agents known to selectively modulate gene expression (1,2). The first antisense product, formivirsen sodium (Vitravene™) an intravitreal injection designed against cytomegalovirus retinitis infections, has recently been approved (3,4). The development of non-parenteral dosage forms for these compounds is desirable. However, the high molecular weight, the hydrophilicity and multiple negative charges result in a poor absorption of antisense oligonucleotides (5). The oral administration faces additional problems such as degradation in the acidic gastric environment, enzymatic metabolism in the lumen and at the gastrointestinal epithelium and first-pass hepatic clearance (6). To achieve a successful non-parenteral delivery of antisense therapeutics, it is necessary to solve the specific problems of the oral administration route, together with general concerns of correct time-space targeting, improved cellular uptake and nuclear localization to exert gene transfection (7,8).

Microparticles made of alginate crosslinked with calcium ions and poly-L-lysine have been reported to effectively protect living tissues from rejection after transplantation (9,10), to accomplish oral and nasal vaccination (11,12), and to increase the metabolic stability of bound antisense oligonucleotides *in vitro* (13). Alginate-based particles have demonstrated biodegradable, biocompatible and mucoadhesive properties (14–16), which are of great interest for the oral delivery of oligonucleotides. The use of a multivalent crosslinking agent such as poly-L-lysine, potentially incorporates the ability of this polycation as transfecting agent (17,18).

The use of permeation enhancers, such as fatty acids and bile salts, is known to facilitate the paracellular permeability of hydrophilic macromolecules through tight junctions (19). The mechanism of action of medium chain fatty acids is related to signal pathways and to the reduction of cellular dehydrogenase activity and ATP levels (20). The disassembly of the tight junctions has been attributed to the dephosphorylation of some proteins involved in the structure, such as occludin (21), the depletion of serosal calcium levels (22) and a surfactant effect (23). In this study, the *in vivo* performance of alginate/poly-L-lysine microparticles formulated in aqueous medium and containing different oligonucleotides and permeation enhancers was evaluated in both rat and dog models after intrajejunal application.

MATERIALS AND METHODS

Materials and Animals

Poly-L-lysine hydrobromide (PLL) with molecular weights of 9600, 9400 and 7500 Da, alginic acid sodium salt (Na-alginate) from Macrocystis pyrifera with medium viscosity (viscosity of 2% solution, 25°C, approx. 3500 cps), calcium chloride anhydrous or dihydrate, tris[hydroxymethyl]aminomethane (Trizma®) hydrochloride, sodium bromide, potassium chloride, acetonitrile, lauric acid sodium salt, capric acid sodium salt, chenodeoxycholic acid sodium salt and ursodeoxycholic acid sodium salt (Sigma Chemical Co., St. Louis, Missouri). Ultrapure Milli-Q® water was used throughout the study. Phosphate buffer solution pH 7.4 was prepared according to the USP XXIII. All other chemicals were reagent grade.

The following phosphorothioate oligodeoxynucleotides (ODN), ISIS 5132 (5′-TCC CGC CTG TGA CAT GCA TT-3′) inhibitor of protein C-raf kinase, ISIS 3521 (5′-GTT CTC GCT GGT GAG TTT CA-3′) inhibitor of C-alpha kinase and ISIS 2302 (5′-GCC CAA GCT GGC ATC CGT CA-3′) designed to inhibit human ICAM-1 (intercellular cell adhesion molecule) expression, were synthesized at Isis Pharmaceuticals,

[1] College of Pharmacy, Freie Universität Berlin, Kelchstr. 31, 12169 Berlin, Germany.

[2] Isis Pharmaceuticals Inc., 2292 Faraday Avenue, Carlsbad, California 92008.

[3] To whom correspondence should be addressed: (bodmeier@zedat.fu-berlin.de)

ABBREVIATIONS: AUC, area under the curve; BAV, bioavailability; EDTA, ethylenediaminetetra-acetic acid; HPLC, high performance liquid chromatography; ICAM-1, intercellular cell adhesion molecule; PBS, phosphate buffer solution; PE, permeation enhancers; PLL, Poly-L-lysine hydrobromide; SAX, strong anionic exchange.

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Inc. following the deoxynucleoside phosphoroamidite method (24).

Male Sprague Dawley rats (200–300 g) were obtained from Harlan Sprague Dawley (Madison, Wisconsin). The rats were housed in polycarbonate cages and had access to rat chow and water *ad libitum* in compliance with IUACCAC guidelines. Male beagle dogs weighing between 12–16 kg were maintained on a standard solid meal of commercial diet and had *ad libitum* access to water. The dogs were housed individually in standard caging meeting USDA regulations (U.S. Department of Agriculture. Animal Welfare. Final Rules. 9 CFR Parts1, 2 and 3. 1995).

Microparticle Preparation

Alginate/poly-L-lysine microparticle formation was achieved by the ionotropic gelation of alginate with calcium chloride followed by crosslinking with poly-L-lysine (25). A 0.09% (w/v) Na-alginate solution (66.7 ml) was treated under magnetic stirring with a 18 mM $CaCl_2$ stock solution (5.0 ml), then deionized water was added (28.3 ml) leading to a Ca-alginate pre-gel with concentrations of 0.06% (w/v) in Na-alginate and 0.9 mM in calcium. 0.3% (w/v) poly-L-lysine solution was added to the Ca-alginate pre-gel under magnetic stirring to form microparticles. The oligonucleotide was dissolved in an aliquot of the supernatant and added back to the particle dispersion, which was equilibrated over 4 d under stirring. Microparticles were separated from the aqueous medium by sedimentation and centrifugation at 3000 rpm for 15 min. The amount of free oligonucleotide in the clear supernatant was determined by UV-spectrophotometry at 260 nm (UV-2101 PC, Shimadzu Scientific Instruments Inc., Columbia, Maryland) in triplicate. The oligonucleotide association capacity was calculated as follows,

$$\% \text{ Association capacity} = 100 \times (\text{total amount of ODN} - \text{free amount of ODN})/(\text{total amount of ODN})$$

After separation, the supernatant was eliminated and the isolated 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.

Determination of the ODN Drug Loading of the Microparticles

To determine the oligonucleotide content, dried microparticles (5–10 mg) were placed into 25 or 50 ml of 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, and agitated for 2 h in a horizontal shaker (IKA HS 501, Janke & Kunkel & Co IKA Labortechnik, Staufen, Germany). Extractions were performed in triplicate. The oligonucleotide concentration was determined by UV-spectrophotometry at 260 nm (UV-2101 PC, Shimadzu Scientific Instruments Inc., Columbia, Maryland). The oligonucleotide loading was calculated as follows,

$$\% \text{ Loading} = 100 * (\text{amount of extracted ODN})/(\text{amount of freeze-dried microparticles})$$

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) directly from the formulation medium before microparticle separation. Lyophilized microparticles (10 mg) were redispersed in different media (50 ml), the particle size distribution was measured with 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 given (n = 3).

Morphologic Characterization by 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 afterwards observed with a scanning electron microscope (Philips SEM 515, PW 6703, Philips Optical Electronics, Eindhoven, Netherlands).

Release Studies

Lyophilized microparticles (approx. 10 mg) were placed into flasks containing 50 ml of release medium at 37°C and shaken horizontally at 75 rpm (GFL 3033, Gesellschaft für Labortechnik, Burgwedel, Germany) over 24 h, in triplicate. The release behavior was determined into water and phosphate buffer pH 7.4, with/out permeation enhancers (PE) consisting of a lyophilized mixture of bile and fatty acid salts, which were added in a proportion of 5:1 (w/w) to the oligonucleotide loaded in the microparticles. Samples of 2 ml were taken at fixed time intervals and replaced with fresh medium. The oligonucleotide concentration was analyzed by UV-spectrophotometry.

In Vivo Rat Studies

After overnight fasting, Sprague-Dawley male rats weighing about 250 g were anesthetized with 5% pentobarbital (50 mg/kg) by intraperitoneal injection. A midline abdominal incision was made to pull out the small intestine and locate the injection site at the beginning of the jejunum, approx. 2 cm after the ligament of Treitz. Each animal (n = 3) received a treatment consisting of alginate-poly-L-lysine-oligonucleotide microparticles redispersed in distilled water with/out a blend of lyophilized PE (bile salts and fatty acid salts), at a dose of 5 mg or 10 mg of oligonucleotide and 50 mg of PE mixture per animal. Afterwards, the intestine was carefully put back into the abdominal cavity. Blood samples of 200–300 μl were collected from the femoral vein 30 min, 1 h, 2 h and 3 h after dosing, transferred to 1.5 ml Eppendorf tubes containing 10 μl of EDTA 0.5 M and centrifuged at 6000 rpm for 6 min for plasma separation. Plasma samples were isolated and kept frozen at –70°C until HPLC analysis. Rats were sacrificed after 24 h and tissue samples were collected. Tissues were washed with saline and fixed with formalin for further immunohistochemistry analysis using monoclonal antibodies against a specific phosphorothioate oligonucleotide (26,27).

The intrajejunal bioavailability (% BAV) was determined using the cumulative area under the curve (AUC) be-

Table I. Composition of the Batches of Alginate/Poly-L-Lysine/Oligonucleotide Microparticles with Specifications for the Poly-L-Lysine/Oligonucleotide Charge Ratio (PLL/ODN +/–), % of Association Capacity and % of Loading

Batch	Na-alginate % (w/v)	Ca mM	PLL μM	PLL MW	ODN μM	ODN MW	ODN ISIS	PLL/ODN (+/–)	Alginate/PLL/ODN (w/w/w)	Association %	Loading %
1	0.05	0.75	52.08	9600	78.77	6347.5	5132	1.60	1:1:1	92.43	29.07
2	0.05	0.75	10.85	9600	78.77	6347.5	5132	0.34	1:0.2:1	31.66	
3	0.05	0.75	52.08	7500	77.70	6435.3	3521	1.26	1:0.8:1	73.50	27.44
4	0.05	0.75	53.20	9400	72.80	6781.3	2302	1.73	1:1:1	95.09	34.97

tween 0 h and 3 h of each plot of plasmatic level vs. time, the dose of pure oligonucleotide normalized to the body weight administered in each experiment, and the AUC of 354 (μg × h/ml) obtained from an intravenous injection of a dose of 10 mg of anhydrous oligonucleotide to 250 g rats, with the following relation,

$$\%\ \mathrm{BAV} = 100 \times [(\mathrm{AUC}_{0\text{-}3}/\mathrm{dose})_{\mathrm{intrajejunal}}/(\mathrm{AUC/dose})_{\mathrm{intravenous}}]$$

In Vivo Dog Study

Male beagle dogs were catheterized for intrajejunal treatment with alginate-poly-L-lysine-oligonucleotide microparticles redispersed in water and lyophilized PE mixture. The dose was 10 mg of anhydrous oligonucleotide and 50 mg of PE blend per kg of body weight. The test formulation, containing 42 mg of microparticles and 65 mg of PE per 1 ml of dispersion medium, was administered (0.77 ml/kg) to overnight fasted beagle dogs (n = 3) through the jejunal catheter as a single bolus injection, followed by a 5 ml water rinse. The water rinsing was repeated several times 30 min after dosing, if necessary. The syringe was weighed before and after administration to control the accuracy of the given dose. Blood samples of approx. 5 ml were collected 10 min, 20 min, 30 min, 45 min and 60 min after dosing via the cephalic or jugular vein, and processed as previously described for rat blood specimens to isolate the plasma fraction for further HPLC analysis. The intrajejunal (% BAV) was determined using the cumulative area under the curve (AUC) over the first hour, the administered dose of anhydrous oligonucleotide normalized to the body weight, and the AUC of 4938 (μg × min/ml) obtained from an intravenous injection of a dose of 10 mg/kg of anhydrous oligonucleotide, with the relation,

$$\%\ \mathrm{BAV} = 100 \times [(\mathrm{AUC}_{0\text{-}1}/\mathrm{dose})_{\mathrm{intrajejunal}}/(\mathrm{AUC/\ dose})_{\mathrm{intravenous}}]$$

Strong Anionic Exchange HPLC

The oligonucleotide concentration in plasma samples was determined by strong anionic exchange HPLC analysis (Waters 625 LC System, Millipore Corporation, Milford, Massachusetts). An aliquot of 80 μl of plasma was diluted with mobile phase B in proportion 1:1 (v/v) prior to analysis. The analytical conditions consisted in an anion exchange column Resource™ Q (1 ml, Pharmacia Biotech AB, Uppsala, Sweden), mobile phase A: 1.0 M NaCl, 0.1 M Na_2HPO_4, mobile phase B: 3.0 M NaCl, 0.1 M Na_2HPO_4, pH adjusted to 11.5 with NaOH, increasing gradient, 1.0 ml/min flow rate,

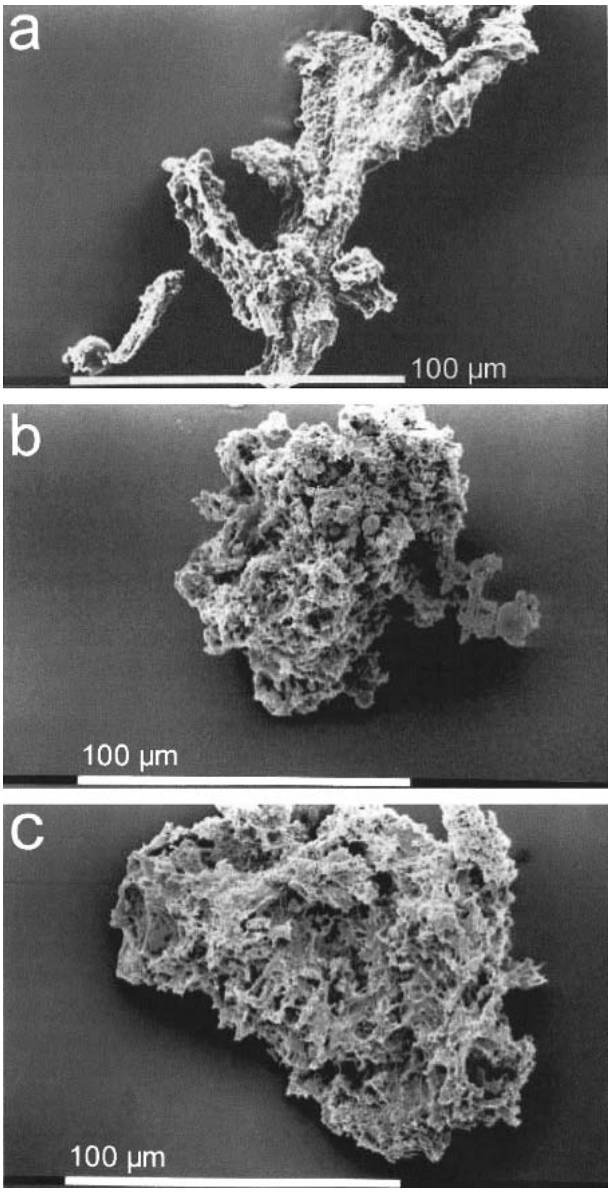

Fig. 1. Scanning electron microscopy (magnification of 6.25 E2, scale 100 μm) of lyophilized alginate/poly-L-lysine/oligonucleotide microparticles from different batches and decreasing amounts of crosslinking agent: (a) microparticles with a PLL/ODN ratio of 1.73 ± corresponding to batch 4; (b) microparticles with a PLL/ODN ratio of 1.60 ± from batch 1 and c) microparticles with a PLL/ODN ratio of 1.26 ± from batch 3.

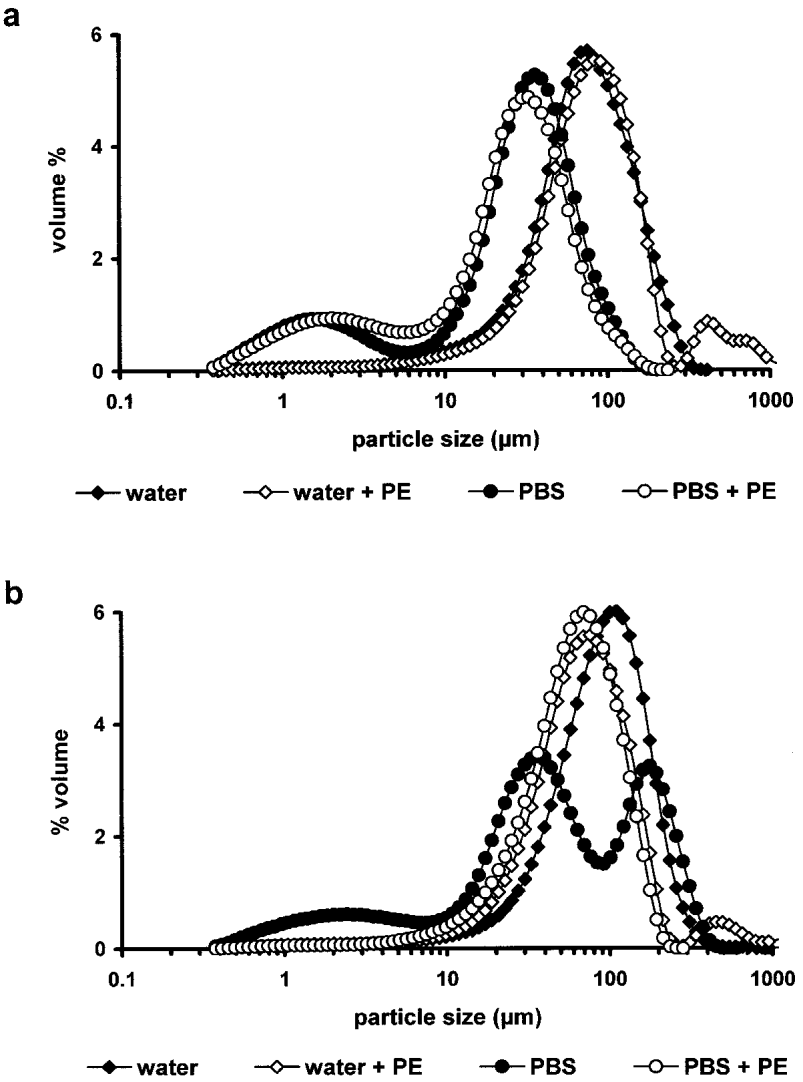

Fig. 2. Effect of the composition of the reconstitution medium, water and phosphate buffer solution (PBS) in the presence and absence of a mixture of permeation enhancers (PE), and time, on the particle size distribution of lyophilized alginate/poly-L-lysine/oligonucleotide microparticles corresponding to batch 4 (a) 2 h after reconstitution and (b) after 1 day.

30 min run time, 30 μl injection volume and UV detection at 260 nm.

RESULTS AND DISCUSSION

Microparticle Formation and Characterization

Oligonucleotide-containing microparticles were obtained from several formulations having differences in the molecular weight and final concentration of the crosslinking agent and the oligonucleotide. Poly-L-lysine had a molecular weight below 10 kDa. The different oligonucleotides had a phosphorothioate chemistry, a length of 20 nucleotides and a molecular weight between 6–7 kDa. The detailed composition and characteristics are reported in Table I.

The association capacities and the loadings were in good agreement with the ratio between poly-L-lysine and oligonucleotide. A higher content of crosslinking agent resulted in higher association and loading values, and in a more effective entrapment of the oligonucleotide, probably due to a stronger electrostatic attraction. The average particle size of freshly prepared systems was approximately 100 μm for all cases except for batch 2, which displayed a smaller particle size of 28 μm since the content of poly-L-lysine in the formulation was the lowest. Microparticle isolation was not possible for batch 2, some of the Ca-alginate pre-gel remained uncrosslinked because of the low proportion of poly-L-lysine in the formulation, which resulted in a continuous film upon lyophilization instead of a particulate system.

The shape of the freeze-dried microparticles was observed by scanning electron micrography (Fig. 1). All batches had an irregular shape and a sponge-like structure. A higher PLL/ODN charge ratio of 1.73 ± (batch 4) led to a more compact structure and larger globules attached to the surface, probably formed by the interaction of more alginate with poly-L-lysine and oligonucleotide (Fig. 1a). Decreasing the proportion of crosslinking agent in the formulation to a PLL/ODN charge ratio of 1.60 ± (batch 1) and of 1.26 ± (batch 3), resulted in a looser and more filamentous particle (Fig. 1b and 1c).

Effect of the Reconstitution Medium on the Particle Size

The microparticles (batch 4) were isolated, lyophilized and sieved through a 160 μm mesh to obtain a free-flowing powder. To study the impact of the medium composition on the particle size of reconstituted systems, the lyophilized microparticles were redispersed into water and phosphate buffer pH 7.4 in the presence and absence of a mixture of permeation enhancers. The amount of permeation enhancers was kept at a proportion of 5:1 (w/w) to the amount of oligonucleotide incorporated within the particulate system. Fig. 2 shows the particle size distributions in the different media, 2 h after reconstitution (Fig. 2a) and after 1 day (Fig. 2b). Microparticles displayed a similar size distribution in water, either with/out permeation enhancers, with a median size of 85 μm and 76 μm respectively. Reconstitution in phosphate buffer led to smaller microparticles with a fraction around 30 μm and another one at about 1 μm. Probably, the phosphate anions interfered with the polyionic polymeric matrix reducing it into smaller microparticles by compacting or by breaking the sponge-like structures due to the higher ionic strength of the medium. In phosphate buffer, the presence of permeation enhancers lightly affected the resulting median size, 27 μm, compared to the case without permeation enhancers with 31 μm. After 1 day, the systems in water showed little change with a small decrease to 73 μm in the presence of permeation enhancers. In phosphate buffer, the particles tended to aggregate, for both cases with/out permeation enhancers.

In Vitro Release Profiles

The reported batches were placed in different media to elucidate the mechanism of release of oligonucleotide from the alginate/poly-L-lysine matrix (Fig. 3). *In vitro* release profiles were influenced by the release medium and to a lesser extent by the batch formulation. All systems showed the same release trend, with: water + permeation enhancers > water > phosphate buffer = phosphate buffer + permeation enhancers. The system containing less poly-L-lysine (batch 3) with a PLL/ODN charge ratio of 1.26 ± (Fig. 3b) showed a fast release in water with and without permeation enhancers.

The release data include the amount of free oligonucleotide released from the microparticles, but do not include the fraction of oligonucleotide, which is potentially released in complex form with poly-L-lysine. Macroscopic observation and particle size distribution (Fig. 2) showed a colloidal fraction when the microparticles were placed in phosphate buffer, both in the presence and absence of permeation enhancers. The presence of phosphate anions preferably displaced alginate from the structure, resulting in the release of complexed oligonucleotide. In water either with/out permeation enhancers, no evidence of complex release was observed. The presence of permeation enhancers as only anions in solution activated the release of free oligonucleotide, probably by displacing the oligonucleotide because of a higher affinity for the poly-L-lysine.

In Vivo Rat Studies

In a preliminary study, the intrajejunal performance of microparticles containing the oligonucleotide ISIS 5132 (batch 1) was evaluated in the absence of a mixture of permeation enhancers and resulted in non-detectable plasma concentrations of oligonucleotide. These results evidenced the need for permeation enhancers, consisting of a lyophilized blend of bile salts (Na-ursodeoxycholate or Na-chenodeoxycholate) and fatty acid salts (Na-caprate and Na-laurate), to obtain an intestinal absorption. Further *in vivo* experiments were therefore conducted in the presence of a mixture of permeation enhancers.

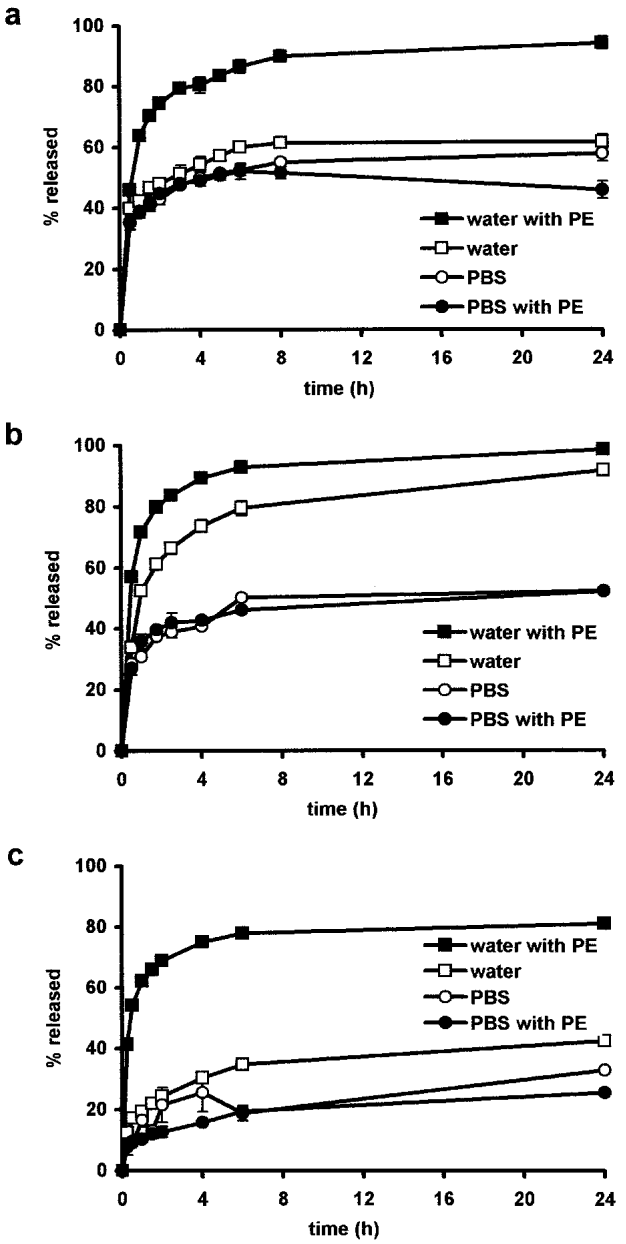

Fig. 3. Release profiles in different media, water and phosphate buffer solution (PBS) in the presence and absence of a mixture of permeation enhancers (PE), of lyophilized alginate/poly-L-lysine/oligonucleotide microparticles corresponding to (a) batch 1, (b) batch 3 and (c) batch 4.

Lyophilized microparticles corresponding to batches 1 and 3 were tested in the same fashion, by intrajejunal instillation of ca. 1 ml of redispersed oligonucleotide-containing microparticles in deionized water with permeation enhancers. Each animal received a dose of approx. 10 mg of oligonucleotide and 50 mg of a blend of permeation enhancers. Batch 3

led to very interesting plasma levels (Fig. 4a) with increasing oligonucleotide concentrations over a 3 h period. On the contrary, the results obtained with batch 1 were much lower (Fig. 4b). The difference between both batches (Table I) was the PLL/ODN ratio. The formulation with a PLL/ODN charge ratio of 1.26 ± (batch 3) led to higher and sustained absorption values compared to that with 1.60 ± (batch 1). A small difference in the PLL/ODN ratio seemed to strongly affect the *in vivo* blood levels while the *in vitro* release profiles were quite similar (Fig. 3a for batch 1 and Fig. 3b for batch 3).

The oligonucleotide tissue distribution was analyzed by immunohistochemistry after intrajejunal administration of microparticles in the presence of permeation enhancers. The localization of the oligonucleotide was possible with a monoclonal antibody that recognized ISIS 3521 (in batch 3) and stained into a dark-brown color. Nuclei were counterstained with hematoxylin. All tested animals were sacrificed 24 h after dosing for tissue collection and showed the same results. A high uptake of the oligonucleotide was observed in the liver into the Kupffer cells (Fig. 5a). Also, strong immunostaining

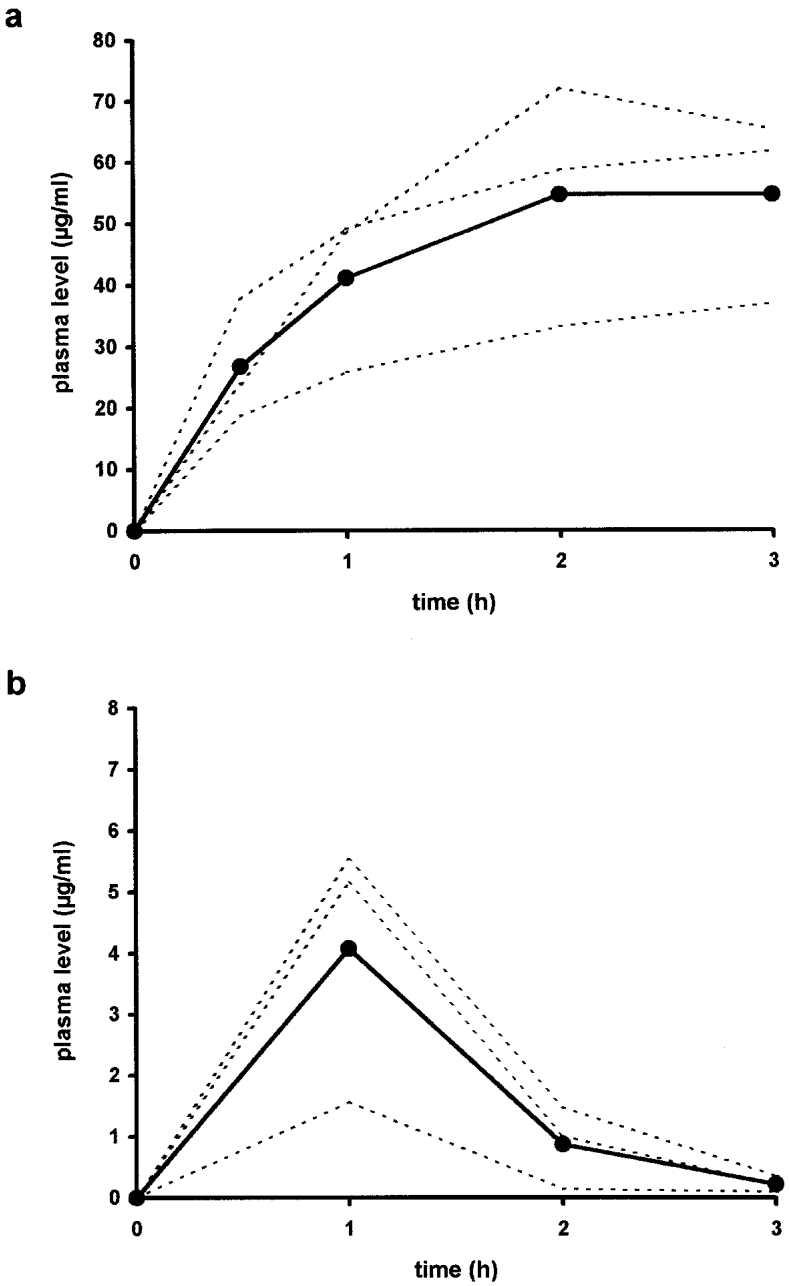

Fig. 4. Concentration profiles of oligonucleotide in plasma after the intrajejunal instillation of lyophilized microparticles in the presence of permeation enhancers in proportion 5 fold the amount of oligonucleotide, following the *in vivo* rat model. (a) Batch 3, alginate/poly-L-lysine/ISIS 3521 microparticles with a PLL/ODN ratio of 1.26 ±. (b) Batch 1, alginate/poly-L-lysine/ISIS 5132 microparticles with a PLL/ODN ratio of ±1.60. The dotted lines correspond to individual rats and the bold line to the mean value (n = 3).

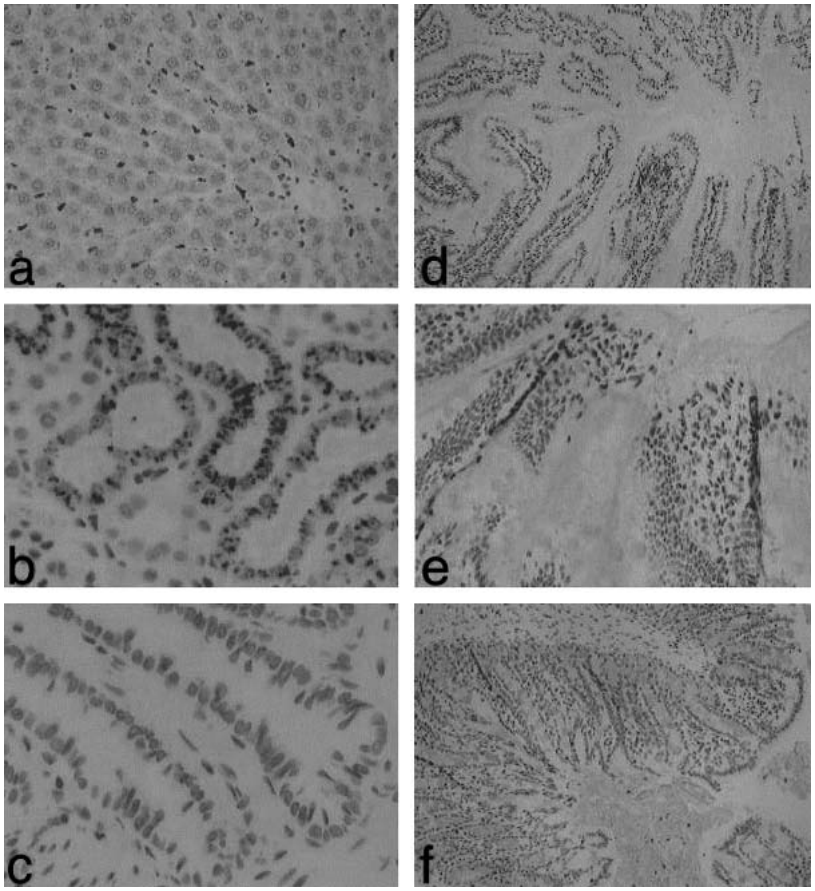

Fig. 5. Immunostaining of rat tissues at 24 h after intrajejunal administration of alginate/poly-L-lysine/ISIS 3521 microparticles (batch 3) in presence of permeation enhancers: (a) liver, (b) kidney, (c) lung, (d) jejunum, (e) ileum and f) colon. Nuclei were counterstained with hematoxylin.

appeared in the proximal convoluted tubules cells of the kidney (Fig. 5b). Kidneys and liver were the organs of major deposition of antisense oligonucleotide after clearance from the systemic circulation. The oligonucleotide was not visible in lungs (Fig. 5c) and duodenum. In the jejunum, high oligonucleotide uptake was localized in the epithelial layer, but in particular, deep in the central lamina propria of jejunal villi (Fig. 5d). This finding showed the absorption of oligonucleotide in the upper small intestine. Some staining was observed at the epithelial level in the ileum (Fig. 5e) and the colon (Fig. 5f). The histologic results indicated a good systemic distribution and in addition, an intimate local association to the intestinal mucosa was observed.

In a separate study, microparticles were formulated as specified for batch 4 and tested for intrajejunal performance in rats in the presence of permeation enhancers. The dose was set at 5 mg of ISIS 2302 and 50 mg of a mixture of permeation enhancers. In this case, the proportion of oligonucleotide to permeation enhancers was decreased from 1:5 to 1:10 (w/w). Oligonucleotide-containing microparticles and permeation enhancers were administered either directly as a powder through an incision at the jejunal level, or redispersed in 1.0 ml of water by intrajejunal instillation. Likewise, free oligonucleotide and permeation enhancers were administered as a powder or as a solution to other groups of test rats. The plasma levels resulting from the four different formulations are shown in Fig. 6. A similar and high plasma profile was observed for both free and formulated oligonucleotide given directly as a powder. In comparison, the oligonucleotide plasma levels were lower when administered as solution or as redispersed microparticles. Probably, the effect of the permeation enhancers onto the intestinal epithelium was stronger in the absence of liquid (lower dilution effect), which promoted the absorption of the oligonucleotide administered in free form or within microparticles. The rate of oligonucleotide dissolution in the intestinal fluids might have matched the effective window of the permeation enhancers in an optimal way. Macroscopic and histologic evaluations of the tissues showed non-damaged tissue structures. From these results, it is possible to point out the necessity of a precise pattern of dissolution, of both the permeation enhancer species and the oligonucleotide from the pharmaceutical dosage form for a successful absorption. It seemed preferable to obtain a high concentration gradient of the permeation enhancers close to the intestinal membrane shortly before or concomitant with the oligonucleotide dissolution. Likely, an amount of permeation enhancers above the critical micellar concentration promoted the thinning of the mucin layer, increased the fluidity of the intestinal membrane and enhanced the paracellular transport by regulation of the tight junctions. Although the incorporation of oligonucleotide within an alginate-based microparticle formulation did not significantly improve the in-

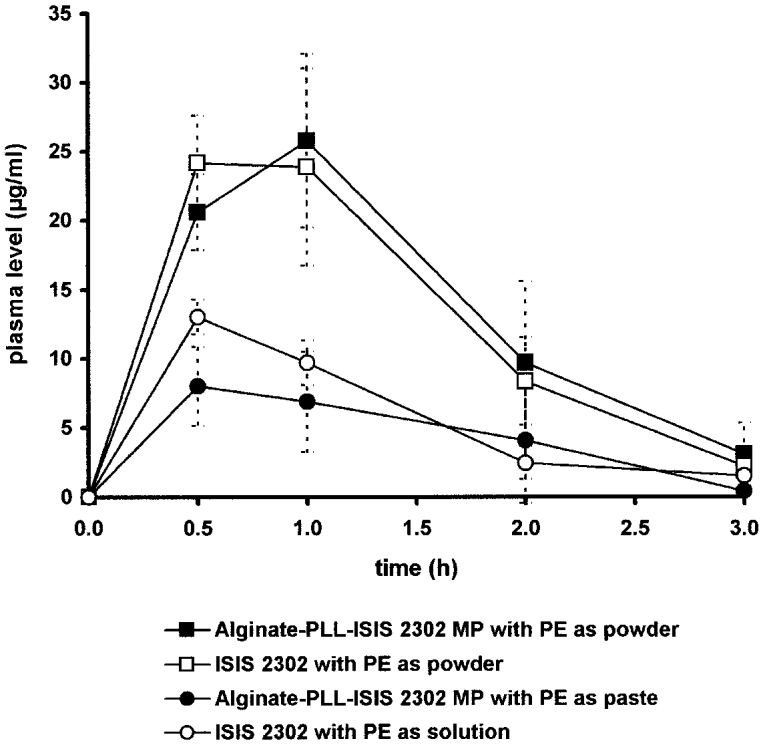

Fig. 6. Rat *in vivo* studies with alginate/poly-L-lysine/ISIS 2302 microparticles (MP) from batch 4 and free ISIS 2302, given as a powder or a fluid form, by intrajejunal application (n = 3). All formulations were tested in the presence of permeation enhancers (PE) mixture in proportion 1:10 (w/w) ISIS 2302 to PE.

testinal bioavailability compared to the administration of free oligonucleotide, it provides a vehicle for the concomitant delivery of the oligonucleotide and the permeation enhancers and, in combination with poly-L-lysine, enzymatic stability in the GI-tract.

Table II summarizes the rat *in vivo* tests and the bioavailability data (% BAV) from each experiment. The rat *in vivo* model only allowed to characterize the plasma concentrations of oligonucleotide over 3 h following intrajejunal administration. Thus, the AUC up to 3 h was used for calculations, without considering the extrapolation to longer times. Therefore, the experimental bioavailability values probably represent a sub-estimation of the actual bioavailability, especially with profiles still displaying high plasma levels after 3h (like batch 3, Fig. 4a). The microparticle formulations led to remarkable bioavailability data, in particular for the treatment with redispersed microparticles from batch 3 where 28.2% of the intrajejunally administered oligonucleotide arrived in the blood stream. The experiments carried out with microparticles from batch 4 and analogously with free oligonucleotide, showed a small increase in the bioavailability of the formulated oligonucleotide (25.4% compared to 22.3% for powder presentations, and 10.4% compared to 9.6% for fluid ones) and a considerable reduction of the absorption when administrated with fluid. The alginate and the poly-L-lysine contained within the microparticle structure might have shown some adhesion onto the intestinal epithelium, thus releasing the oligonucleotide closer to the absorption site and over a sustained period. The ideal administration will comprise a powder microparticle formulation within an enteric resistant solid dosage form that disintegrates at the upper small intestine; thus favoring the contact with the mucosa, moisture uptake and the activation of the release of oligonucleotide where absorption occurs.

Table II. Summary of the *in Vivo* Rat Tests with the Administered Formulation and the Obtained Bioavailability (% BAV) after Intrajejunal Application

Test formulation	MP batch	ODN	ODN:PE (w/w)	% BAV
Redispersed MP with PE	1	ISIS 5132	1:5	2.11 (± 0.99)
Redispersed MP with PE	3	ISIS 3521	1:5	28.17 (± 7.27)
Powder MP with PE	4	ISIS 2302	1:10	25.39 (± 7.15)
Powder ODN with PE		ISIS 2302	1:10	22.30 (± 5.99)
Redispersed MP with PE	4	ISIS 2302	1:10	10.36 (± 4.74)
Solution ODN with PE		ISIS 2302	1:10	9.61 (± 1.23)

Note: MP microparticles, ODN oligonucleotide, PE permeation enhancers mixture.

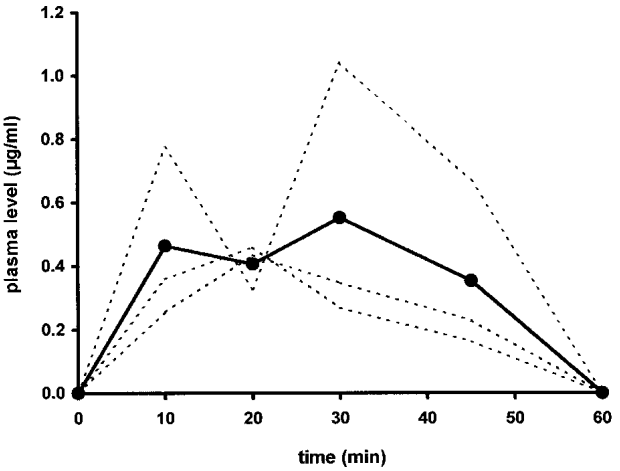

Fig. 7. Dog *in vivo* studies with alginate/poly-L-lysine/ISIS 2302 microparticles from batch 4 administered with permeation enhancers in proportion 5 fold the amount of oligonucleotide via intrajejunal catheter. The dotted lines correspond to ISIS 2302 plasma levels of single dogs and the bold line with the mean value (n = 3).

In Vivo Dog Studies

Oligonucleotide-containing microparticles from batch 4 and a lyophilized mixture of permeation enhancers were redispersed together and administered to beagle dogs via intrajejunal catheter, at a dose of 10 mg/kg of anhydrous ISIS 2302 and 50 mg/kg of a mixture of permeation enhancers. The concentrations of ISIS 2302 in plasma were recorded during the first hour following administration. Fig. 7 illustrates the plasma level-time profiles for each individual (dotted line) and the mean (thick line), which resulted in low oligonucleotide concentrations. Only approx. 0.42% of the dose reached the systemic circulation, in contrast to what was expected from the *in vivo* rat studies. Further investigations could optimize the dog model by modifying the dosage form (i.e. powder instead of fluid) and the site of administration, thus identifying a potential absorption window and optimizing the time sequence for blood sampling.

CONCLUSIONS

An antisense oligonucleotide carrier of alginate/poly-L-lysine microparticles was developed in an aqueous environment, using mild conditions, based on the ionotropic gelation of alginate. Sponge-like microparticles were obtained at different proportions between components, with high association capacities and loadings. The *in vitro* release behavior depended mostly on the medium composition, which revealed the importance of competitive anions and ionic strength on the mechanism of dissociation of the oligonucleotide from the polymeric matrix. Rat *in vivo* studies showed promising oligonucleotide bioavailability for microparticles based on alginate and poly-L-lysine, after intrajejunal administration in the presence of a mixture of permeation enhancers. The results and the complexity of the formulation evidenced a subtle harmony, between the concentration of permeation enhancers at the absorption site and the release of oligonucleotide from the pharmaceutical form, to achieve a successful intestinal application.

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