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Albumin nanoparticles improved the stability, nuclear accumulation and anticytomegaloviral activity of a phosphodiester oligonucleotide

A. Arnedo, J.M. Irache, M. Merodio, M.S. Espuelas Millán*

Centro Galénico, Departamento de Farmacia y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de Navarra, Irunlarrea 1, Ap. 177, 31080 Pamplona, Spain

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Abstract

The goal of this study was to evaluate the potential of albumin nanoparticles as a delivery system for antisense oligonucleotides. Nanoparticles were prepared by a coacervation process and cross-linkage with glutaraldehyde. Phosphodiester (PO) and phosphorotioate (PS) oligonucleotides were either adsorbed on the surface of nanoparticles (PO-NPA and PS-NPA) or incorporated in the nanoparticle matrix (PO-NPB and PS-NPB). When PO-loaded nanoparticles were incubated with phosphodiesterase, only NPB was able to keep the oligonucleotide hybridization capability for at least 60 min. The antiviral activity was evaluated in MRC-5 fibroblasts infected with human cytomegalovirus at a MOI of 0.0035. Both PO nanoparticle formulations significantly increased the antiviral activity of free PO ($P<0.001$) and NPB showed slightly higher efficacies than NPA ($P<0.05$). On the other hand, PS exhibited significant higher activity than free PO ($P<0.001$), however, no significant differences were found between PS-nanoparticle and PO-nanoparticle formulations. These findings were well correlated with the intracellular distribution observed for fluorescent oligonucleotide-loaded albumin nanoparticles. Even these carriers delayed and decreased the uptake of PO by MRC-5 cells, they finally induced a diffused cytoplasmic distribution and major nuclear accumulation. In summary, albumin nanoparticles partially protected a PO against enzymatic degradation and improved their presence in the nucleus and thus, increased its efficiency.

Keywords: Albumin nanoparticles; Oligonucleotide; Cellular uptake; Enzymatic stability; Antiviral

1. Introduction

Antisense oligonucleotides are promising agents that may be widely used in the regulation of inappropriate expression of genes in pathological situations by specific inhibition of the expression of their mRNA targets [1]. However, the widespread use of these molecules in clinic has been limited by two major obstacles, their enzymatic instability [2] and poor and inadequate intracellular delivery [3]. Oligonucleotide seems to be internalized through an endocytic mechanism [4]. This sequestration of oligonucleotides into endosomal compartments means their degradation, impairing the interaction with their target mRNA and hence decrease their activity [5].

* Corresponding author. Tel.: +34-948-42-5600; fax: +34-948-42-5649.
E-mail address: sespuelas@unav.es (M.S. Espuelas Millán).

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Several strategies have been developed to solve these problems and improve the oligonucleotide efficacy. One of them is the use of chemically modified oligonucleotides (i.e. phosphorotioate), which enhances their stability against enzymatic degradation. Another possibility may consist in the use of colloidal carriers that protect unmodified oligonucleotides against enzymatic degradation and also improve their cellular uptake. In this way, liposomes with cationic lipids have been the most widely exploited [6]. However, this type of carrier has unfavorable pharmacokinetic for particularly most in vivo applications, because of its high toxicity and interaction with serum components. Recently, biodegradable nanoparticles have also been studied as potential inert and biocompatible carriers for genetic materials v.g. polylactide polyethylene glycol (PLA-PEG) [7], cationic polystyrene [8], chitosan [9], spongelike alginate [10], polyalkylcyanoacrylate [11], polyisohexylcyanoacrylate [12] or polyisobutylcyanoacrylate [13] nanoparticles. In the most cases, the coating of these particles with cationic polymers was required to obtain successful interactions with the negatively charged ON molecules [8,12,14]. Other strategy was the covalent linkage of the ON to a hydrophobic molecule to allow the hydrophobic interaction with polymer surface [15].

The cellular uptake of oligonucleotides was dramatically increased when associated with nanospheres [16]. However, whether the ON are used in their free form or in association with these carriers, their internalization involves endocytosis and thus degradation by lysosomal nucleases. Therefore, several compounds able to destabilize the lysosomal membrane were also inserted in liposomes or nanoparticles. Such combination of strategies have been widely studied with lipidic formulations, e.g. DOPE, pH-sensitive liposomes [17] or virosomes. However, at present, fusogenic nanoparticles have been scarcely developed [7,12,18,19].

In a previous work [20], we have shown the ability of albumin nanoparticles to load appreciable amounts of a 21-mer phosphodiester oligonucleotide, either adsorbed onto pre-formed nanoparticles or encapsulated inside the matrix of the carriers. As advantage as compared with other nanoparticles designed for ON delivery, the association of the ON with albumin carriers did not require the addition of any positive compounds. In fact, the plasma albumin is the main endogenous carrier for polyanions and such interaction is spontaneous. What is more, we have recently observed [21] that the complexation of this oligonucleotide with bovine serum albumin (BSA) improved its nuclear accumulation and thus, its biological activity. This fact could be tightly associated with the fusogenic properties previously described for serum albumin at low pH. The protein induced the fusion of small unilamellar vesicles [22] and improved the gene delivery into cells cytoplasm [23].

In the present work, we evaluated if albumin nanoparticles could accumulate the oligonucleotide into the nucleus, as described for the native protein [21], in addition to the increase in the oligonucleotide enzymatic stability previously reported [20]. The resulting in vitro biological activity was thus tested in the current study. For this application, an oligonucleotide phosphodiester (PO) and its phosphorotioate analog (PS) were encapsulated into nanoparticles, either during the particle production or by adsorption post-particle preparation. The phosphorotioate analog used, called ISIS 2922 (Vitravene®) has been approved by the FDA advisory committee for the treatment of cytomegalovirus infection in 1998 [24].

2. Materials and methods

2.1. Materials

Phosphodiester (PO), ISIS 2922 (PS), 5′-fluorescein isothiocyanate-loaded phosphodiester (FITC-PO) and 5′-fluorescein isothiocyanate-loaded phosphorotioate (FITC-PS) oligonucleotides with sequence 5′-GCGTTTGCTCTTCTTCTTGCG-; sense sequence 5′-CGCAAGAAGAAGAGCAAACGC- and non-complementary control phosphodiester oligonucleotide sequence 5′-GCGTTCTTCTTCTCGTTTGCG- were provided by Pharmacia Biotech (Cambridge, UK). BSA (fraction V), methanol and chlorhydric acid were purchased from Merck (Darmstadt, Germany). Glutaraldehyde (grade II, 25%), sodium hydroxide, Trypan Blue dye, copper (II) sulfate, bicinchoninic acid solution and proteinase K were obtained from Sigma (Madrid, Spain). Ethanol absolute from Prolabo (Fontenay, France). Hoechst 33342 (DNA fluorescent marker) and SYBR® Green I nucleic acid gel stain were provided by Molecular Probes (Eugene, OR,

USA). β-Gal Assay Kit Invitrogen® was purchased from Invitrogen (De Schelp, The Netherlands) and Crystal violet was by Panreac (Barcelona, Spain). MEM-glutamax, foetal bovine serum (FBS), penicillin–streptomycin (50 U/ml), hepes, non-essential aminoacids (NEAA), trypsin-EDTA and phosphate buffer saline (PBS) were purchased by Gibco-BRL (Spain).

2.2. Preparation and characterization of oligonucleotide-loaded albumin nanoparticles

Nanoparticles were prepared by a coacervation process and cross-linkage with glutaraldehyde. Phosphodiester and phosphorotioate oligonucleotides were either adsorbed or incorporated in albumin nanoparticles following two different procedures [20]. In the former, oligonucleotide-loaded nanoparticles were obtained by the incubation of the antisense oligonucleotide with pre-formed nanoparticles (PO-NPA or PS-NPA). Briefly, nanoparticles were obtained by the addition of 2 ml ethanol to 1 ml aqueous solution of BSA (2% w/v), adjusted to a pH of 5.5 with HCl 0.1 N. Coacervates thus obtained were then hardened with glutaraldehyde (1.56 μg/mg BSA, previously dissolved in ethanol) for 2 h at room temperature. After ethanol elimination by evaporation under reduced pressure (Büchi waterbath B-480, Switzerland), nanoparticles were purified by centrifugation at 17 000 rpm for 30 min (Sorvall RC-plus, rotor SS-34, Connecticut, USA). The oligonucleotide-NPA formulations were then obtained by resuspending the purified nanoparticles with an aqueous solution containing 60 μg/ml of the oligonucleotide and incubating for 2 h at room temperature. In the latter, the oligonucleotide (100 μg/ml) was firstly incubated with the albumin aqueous solution (2% w/v; pH 5.5) for 2 h at room temperature. Then, nanoparticles (PO-NPB and PS-NPB) were obtained as described above.

The size and zeta potential of the different batches were measured at room temperature with a scattering angle of 90° in a Zetamaster instrument (Malvern Instruments, UK) after diluting with distilled water. The amount of protein transformed into nanoparticles (yield) was determined by a standard BCA protein assay [20]. The drug loading was evaluated by fluorimetry in a Cytofluor 2350 (Fluorescence Measurement system, Millipore) (485 λ-absorption/530 λ-emission). The amount of FITC-PO or FITC-PS was assessed in the supernatant after sample centrifugation and supernatant dilution in NaOH 1 M. The drug loading was calculated as the ratio between the amount of drug in nanoparticles (expressed in μg) and the albumin nanoparticles yield (expressed in mg).

2.3. Hybridization capability of loaded oligonucleotide

PO or PS oligonucleotides were extracted from nanoparticles by degradation of the macromolecule matrix with 0.1 mg/ml of proteinase K at 50 °C for 2 h and the hybridization capability of the extracted oligonucleotide was determined using a fluorescence temperature cycler (Lightcycler, Roche Diagnostics, GmbH, Germany).

For this purpose, an aliquot obtained after complex degradation containing 20 ng of antisense oligonucleotide was placed in a glass capillary containing 20 ng of its complementary sense sequence, 1 μl SYBR® Green (1:1000) and buffer 100 mM Tris–HCl (pH 8.9), 100 mM NaCl and 14 mM $MgCl_2$. Sense and antisense oligonucleotides formed a duplex spontaneously by incubation, which bind the SYBR® Green I. This dye emitted a fluorescence signal only when it is bond to the duplex double stranded. The fluorescence signal is proportional to the hybridization capability. A melting curve was acquired by the slow heating of the duplex at 0.2 °C/s to 95 °C, measuring fluorescence absorbance during the process (with a total of 300 points per run). For improved visualization of the Tm (temperature at which 50% of the originally added PO is still in a duplex [25], melting peaks were derived from the initial melting curves (fluorescence [F] vs. temperature [T] by plotting the negative derivative of fluorescence over temperature vs. temperature ($-[dF/dT]$ vs. T).

2.4. Stability of oligonucleotide in the presence of phosphodiesterase

PO or PS oligonucleotides (2.5 μM), free or associated to albumin nanoparticles, were incubated with snake venom phosphodiesterase (0.01–0.25 mg/ml) in buffer 100 mM Tris–HCl (pH 8.9), 100 mM NaCl and 14 mM $MgCl_2$. These experiments were

carried out in a shaking bath at 37 ± 1 °C with a constant agitation of 60 strokes per min (Unitronic 320 OR, Selecta, Madrid, Spain). The higher enzyme concentration (0.25 mg/ml) was used as a positive control for the rapid degradation of oligonucleotides, as described by other authors [26]. At different time points, the degradation was stopped by heating the sample at 80 °C for 5 min. Then, samples were incubated with 0.1 mg/ml of proteinase K at 50 °C for 2 h. Turbidity measurements indicated complete solubilization of nanoparticles after this time (data not shown). Therefore, the hybridization capability of the oligonucleotide was determined using a fluorescence temperature cycler (Lightcycler, Roche Diagnostics) as described before. On the other way, samples of free oligonucleotide and nanoparticle-associated oligonucleotide were incubated at 37 °C in 100 mM Tris–HCl (pH 8.9), 100 mM NaCl and 14 mM $MgCl_2$ overnight, and the same post-incubation procedure was applied to check that these conditions were not destructive for the oligonucleotide.

2.5. Cell culture

The cell line of human embryonic lung fibroblasts, MRC-5, was obtained from the European General Cell Collection. The cells were cultured in MEM supplemented with glutamax® (2 mM), 10% FBS, 1% penicillin–streptomycin, 1% NEAA and 10 mM Hepes at 37 °C in a 5% CO_2 humidified incubator. Cells were seeded in tissue culture plates (Costar Corp., Cambridge, USA) and allowed to adhere to them overnight.

2.6. Virus and cell infection

The human cytomegalovirus RC 256 was obtained from the American type culture collection (ATCC) (Rockville, MD), prepared in aliquots and stored at −80 °C until use. RC 256 is a recombinant derivative of the attenuated Towne strain, which carries and expresses the *Escherichia coli* lacZ gene. The titer of the virus stocks determined by plaque reduction assay, near 1.6×10^5 pfu/ml. Cell monolayers, seeded on 24-well tissue culture plates, at confluence, or fibroblast trypsinized at concentration 1×10^4 cell per ml seeded on 96-well tissue culture plates were infected with varying viral titers. Finally, the infected cells were treated according to the assay.

2.7. Antiviral activities of albumin oligonucleotide formulations: plaque formation assay

MRC-5 fibroblasts were seeded in 24-well plates at a density of 5×10^4 cells per cm^2. When the cells reached 70% of confluence, the medium was removed and the cells infected with 200 pfu per well diluted in 50 μl medium supplemented with 2% FBS (multiplicity of infection: 0.0035). Following centrifugation at 1200 rpm for 60 min and incubation at room temperature for 1 h, the inoculum with the non-adsorbed virus was removed. The cells were washed with medium and treated with the oligonucleotide-loaded formulations for 3 days and then, replaced with fresh medium. On day 9 post-infection, the monolayers were fixed with acetone:methanol (1:1) at 4 °C, stained with crystal violet (10%), and scored for plaques with a light microscope. Then, the antiviral activity was calculated as the ratio between the number of plaques in the control wells (untreated wells), and the surviving plaques in the presence of the oligonucleotide formulations. This activity was expressed as percentage of plaques. These experiments were carried out six times for each concentration tested (0.1–10 μM) and subjected to statistical analysis employing ANOVA test for variability and Dunnet when significant differences were found ($P < 0.01$).

2.8. Cellular internalization of albumin oligonucleotide formulations

For the visualization of the oligonucleotide internalization by fibroblasts, MRC-5 were seeded in Lab-Tek® culture plates (1×10^4 cell per cm^2). After overnight, the supernatants were discarded and the FITC-loaded oligonucleotide formulations (5 μM in all cases) in serum-free medium, were added to each well. After incubation at 4 or 37 °C, for 4 or 24 h, the medium was discarded, the cells washed three times with PBS and fixed with methanol at 4 °C. Cellular fluorescence was visualized using a fluorescence microscope (Nikon, FFD-3, Japan) fitted with a Chroma #83490 FITC filter (494 λ-absorption/518 λ-emission). Images were analyzed using the program MacProbe 4.11.

3. Results

3.1. Characterization of oligonucleotide-loaded nanoparticles

The main physico-chemical characteristics of the different oligonucleotide-loaded albumin nanoparticles used in this study are summarized in Table 1. Similar size and zeta potential were observed for all nanoparticle systems; 250 nm and −22 mV, respectively. However, albumin incubated with oligonucleotide before desolvation (PO-NPB, PS-NPB) showed significantly higher drug loadings and encapsulation efficiencies ($P<0.05$) than pre-formed nanoparticles (PO-NPA, PS-NPA). Previous studies also revealed that these nanoparticle carriers show a different release profile. A quick and complete burst for NPA and a byphasic release pattern, with a rapid initial step followed by a sustained and slower release of the loaded drug for NPB were observed [20]. On the other hand, PS displayed a higher affinity for albumin nanoparticles than PO. Thus, NPA were able to load near 3.4 ± 0.2 μg PS oligonucleotide/mg protein, meanwhile a loading of 0.76 ± 0.1 μg/mg protein was obtained for PO. Similarly, for oligonucleotide entrapped in nanoparticles (NPB), the loading of PS and PO were 5.2 ± 0.1 and 4.7 ± 0.1 μg oligonucleotide/mg nanoparticle, successively (Table 1).

3.2. Hybridization capability of loaded oligonucleotide

To ascertain if the process of nanoparticle preparation induced oligonucleotide cleavage, the hybridization capability of the oligonucleotide loaded in albumin carriers was examined. The melting curve demonstrated that PO, either free or associated to albumin nanoparticles, showed similar Tm values. Therefore, the hybridization capability of unmodified oligonucleotide remained intact after nanoparticle preparation (Table 2). Similarly, no difference was found between free PS or PS loaded into albumin particles. However, all phosphorotioate oligonucleotide batches showed lower Tm values than unmodified oligonucleotide formulations (Table 2).

Table 1
Physicochemical characteristics of albumin nanoparticles

Formulation	Size (nm)	Zeta potential (mV)	Drug loading (μg drug/mg NP)	Entrapment efficacy (%)
Unloaded NP	258 ± 1	−24.8 ± 3.0	–	–
PO-NPA	248 ± 5	−22.1 ± 0.3	0.75 ± 0.11	15.94 ± 1.81
PO-NPB	246 ± 6	−21.6 ± 0.9	4.67 ± 0.27	58.94 ± 2.63
PS-NPA	253 ± 2	−23.4 ± 0.3	3.35 ± 0.22	67.50 ± 2.74
PS-NPB	267 ± 7	−21.1 ± 0.4	5.24 ± 0.17	73.56 ± 1.36

Data express the mean ± S.D. ($n=6$).

Table 2
Hybridization capability of oligonucleotides free or loaded into albumin particles by duplex melting temperature determinations

Formulation	Tm values (°C)
PO	63.3 ± 0.1
PO-NPA	65.1 ± 0.5
PO-NPB	65.2 ± 0.8
PS	55.8 ± 0.1
PS-NPA	56.0 ± 0.1
PS-NPB	55.5 ± 0.3

Data express the mean ± S.D. ($n=3$). PO, unmodified oligonucleotide; PS, phosphorotioate oligonucleotide.

3.3. Stability of oligonucleotides in the presence of phosphodiesterase

Fig. 1 shows unmodified oligonucleotide (PO) degradation experiment in presence of phosphodiesterase at concentration 0.01 mg/ml. Free oligonucleotide (PO) lost its hybridization capability in the first minute (data not shown). However, when the oligonucleotide was adsorbed onto nanoparticles, 30 min were required to its complete degradation even if its hybridization capability was really affected in the first minute (Fig. 1B). On the contrary, the hybridization capability of PO entrapped into NPB was completely maintained for 60 min (Fig. 1A). At 0.1 mg/ml phosphodiesterase, PO-NPB formulation maintained the oligonucleotide Tm values during at least 60 min (Table 3). However, free PO or adsorbed on NPA lost its hybridization capability in the first 5 min.

Its phosphorotioate analogue, free PS (Tm = 55.8 ± 0.2), was only partially degraded after incubation with the enzyme overnight (Tm = 44.0 ± 0.4) (Table 3). However, PS-loaded nanoparticles retained its hybridization capability after incubation with phosphodiesterase overnight.

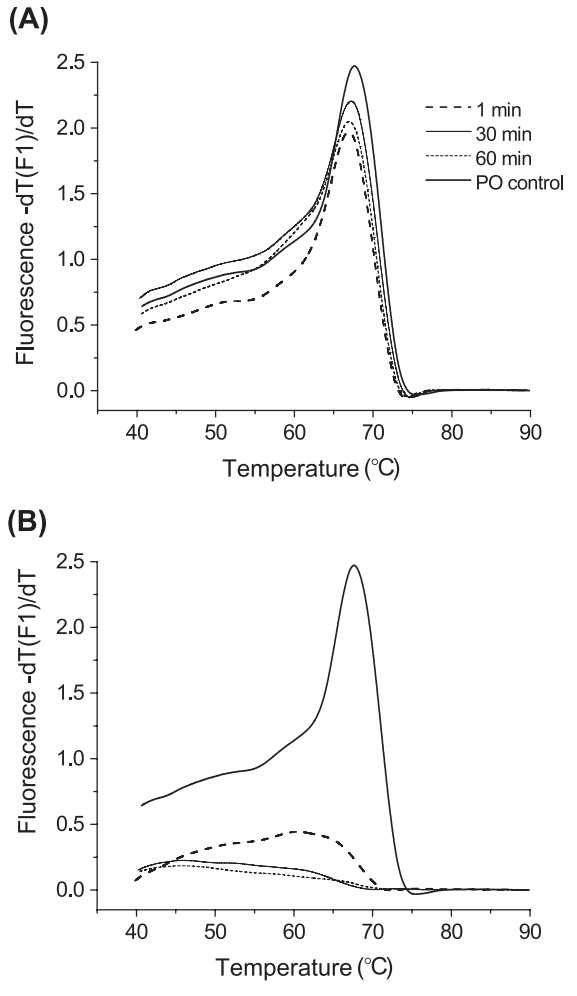

Fig. 1. Degradation experiment of phosphodiester oligonucleotide nanoparticle formulations in the presence of phosphodiesterase 0.01 mg/ml. Control oligonucleotide was free PO without enzyme, but with the same post-incubation procedure than the other batches. (A) No differences in the Tm value were observed between the control PO and PO-NPB after 1, 30 and 60 min of incubation with phosphodiesterase. (B) A rapid lost of hybridization capability was observed for PO-NPA when incubated with phosphodiesterase for 1, 30 or 60 min.

3.4. Antiviral activity of oligonucleotide-loaded nanoparticles: plaque formation assay

Fig. 2 shows the anti-HCMV activity of the different formulations in infected MRC-5 cells, as a function of the oligonucleotide concentration, when added to the cell cultures immediately after viral infection. The non-complementary phosphodiester oligonucleotide (GCGTTCTTCTTCTCGTTTGCG), albumin (data not shown) and empty nanoparticles used as negative controls exhibited reduced antiviral activity relative to that of complementary oligonucleotide.

Free unmodified oligonucleotide (PO) showed a very low antiviral activity. Thus, at 10 μM (high concentration), plaques were still 79.8 ± 4.2% of the control, the efficacy was only about 20% of the control. Both PO nanoparticle formulations significantly increased the antiviral activity of free PO ($P < 0.001$) and their IC_{50} was calculated around 10 μM. Between both types of nanoparticle formulations, NPB showed slightly higher efficacies than NPA ($P < 0.05$) and thus, at concentration 10 μM, PO-NPB decreased HCMV plaques till 43.2 ± 3.2% of the control whereas PO-NPA produced 50.0 ± 6.4% of reduction (Fig. 2A).

Fig. 2B shows the influence of the PS concentration on the HCMV replication. Free PS exhibited significant higher activity than free PO ($P < 0.001$), however, no significant differences were found between PS-nanoparticle and PO-nanoparticle formulations. Neither differences in activity were found between PS free or formulated and it was found similar for all formulations: at concentration 10 μM, the HCMV plaques for PS-NPA were reduced till 49.8 ± 1.6, 53.4 ± 1.6 for PS-NPB and 56.4 ± 5.7% for free PS.

3.5. Oligonucleotide internalization

MRC-5 cells were incubated for 4 or 24 h at 4 or 37 °C with free FITC-PO or FITC-PO entrapped into albumin nanoparticle matrix (PO-NPB) in serum-free medium.

Table 3
Hybridization capability of oligonucleotides, free or loaded into albumin particles, in the presence of snake venom phosphodiesterase (0.1 mg/ml)

Formulation	5 min	60 min	Overnight
PO	Nh[a]	Nh	Nh
PO-NPA	Nh	Nh	Nh
PO-NPB	66.0 ± 0.1	63.9 ± 0.1	Nh
PS	Nd[b]	51.6 ± 0.6	44.0 ± 0.4
PS-NPA	Nd	55.5 ± 0.1	54.6 ± 0.1
PS-NPB	Nd	56.6 ± 0.1	55.0 ± 0.1

Data express the mean ± S.D. ($n = 6$). PO, unmodified oligonucleotide; PS, phosphorotioate oligonucleotide.

[a] Nh, no hybridization capability.

[b] Nd, not determined.

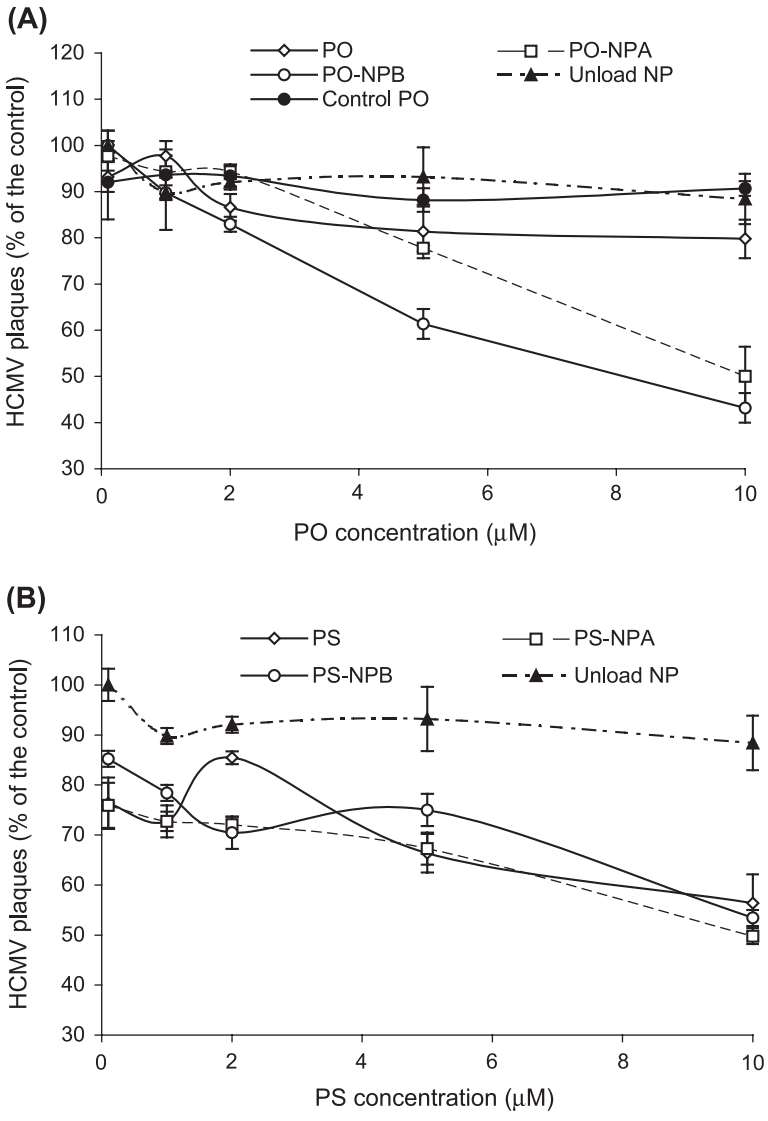

Fig. 2. Effect of the different formulations of oligonucleotides in the HCMV replication on MRC-5 cells by plaque reduction assay. The cells were infected with a MOI of 0.0035 in media 2% FBS. Activity of unloaded nanoparticles is shown for comparison. Error bars represent calculations of standard error on the basis of six times determinations. (A) Unmodified oligonucleotide formulations. (B) Phosphorotioate oligonucleotide formulations.

For free FITC-PO, a perinuclear punctuate pattern corresponding to oligonucleotide accumulation within vesicular compartments such as endosomes and/or lysosomes, with no detectable nuclear accumulation, were observed in all cases (Fig. 3A, C, E). However, an important fluorescence decrease was observed at 4 °C (Fig. 3A) as compared with 37 °C (Fig. 3C) that indicated an uptake of the oligonucleotide by energy-dependent mechanism as endocytosis. The fluorescence intensity decreased with the time (4 vs. 24 h) (Fig. 3C, E), probably caused by a rapid oligonucleotide degradation.

PO entrapped into albumin nanoparticles showed a punctuate homogeneous fluorescence after incubation for 4 h, at 4 and 37 °C (Fig. 3B, D), indicating a simple adsorption of nanoparticles onto the cellular

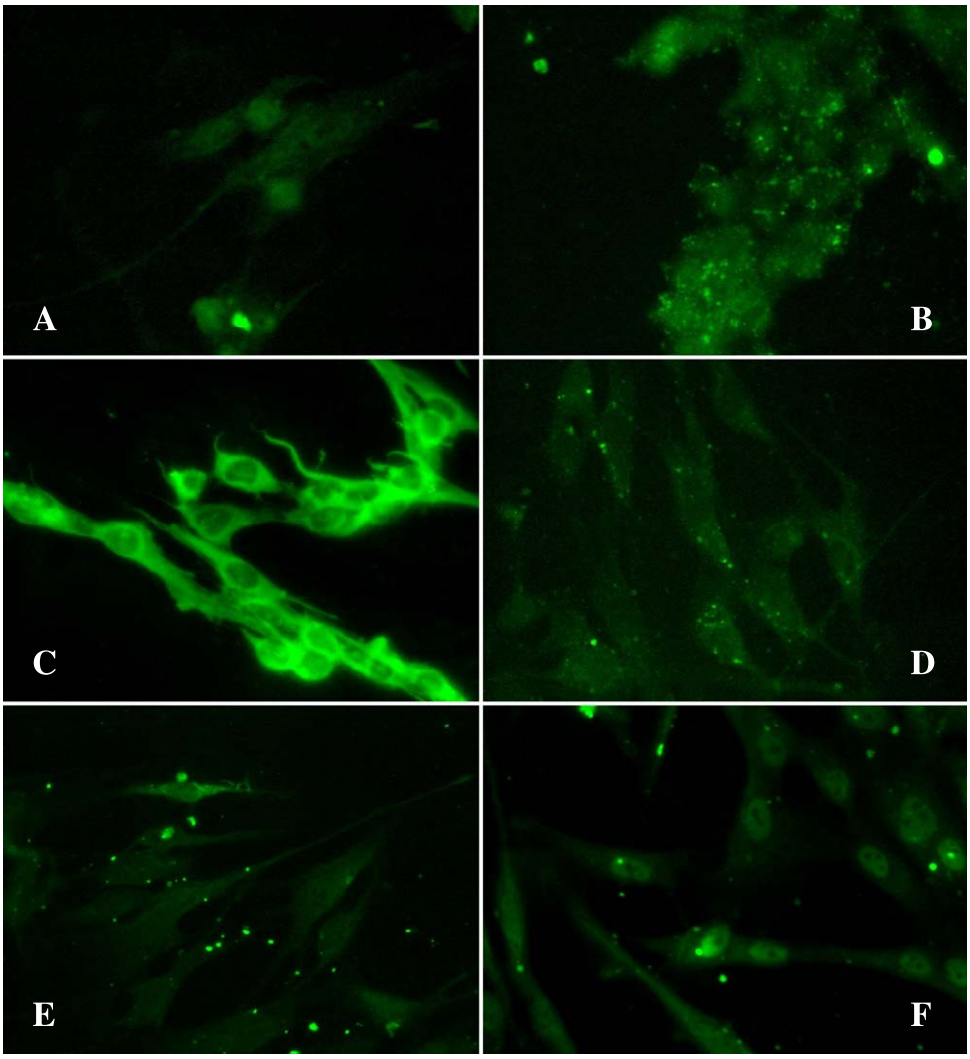

Fig. 3. Subcellular distribution pattern of FITC-PO in non-infected MRC-5 cells by fluorescence microscopy. The cells were incubated with 5 μM oligonucleotide for either 4 or 24 h at 4 or 37 °C. (A) FITC-labeled PO 4 h at 4 °C; (B) FITC-labeled PO-NPB, 4 h at 4 °C; (C) FITC-labeled PO 4 h at 37 °C; (D) FITC-labeled PO-NPB, 4 h at 37 °C; (E) FITC-labeled PO 24 h at 37 °C (F) FITC-labeled PO-NPB 24 h at 37 °C.

surface. However, after 24 h incubation at 37 °C, the fluorescence was homogenous for all the cytoplasm and mainly located in the nucleus (Fig. 3F).

4. Discussion

In the current study, we shown that the association of a phosphodiester oligonucleotide with albumin nanoparticles protected a phosphodiester oligonucleotide against enzymatic degradation and improved its nuclear accumulation. As a consequence, the anti-cytomegaloviral potency of the free oligonucleotide was increased. The contribution of both factors in the observed biological activity are discussed below.

Fluorescence microscopy revealed that, as the native protein [21], BSA nanoparticles greatly decreased the cellular uptake of a phosphodiester oligonucleotide (Fig. 3). The only difference was these carriers slowed down the uptake of the oligonucleo-

tide by cells. This delay could be due to the inability of fibroblasts for endocytic process of nanoparticles (250 nm) (phagocytosis) that thus must be degraded for the released PO to be internalized. However, after 24 h, the oligonucleotide was diffused throughout the whole cytoplasm and mainly located in the nucleus, the location required to exert its antiviral activity. Some nanoparticles have been engineered to promote endosomal escape of oligonucleotides. V.g. a cationic surfactant CTBA was used to coat polisobutylcyanoacrylate [14] or poly(DL-lactic acid) nanoparticles [18] and the authors suggested this cation could destabilize the endosomal membrane. General and Thunemann (2001) [19] described the preparation of pH-sensitive nanoparticles of poly(amino acid) dodecanoate complexes. Cationic hydrophobic peptides were used to complex oligonucleotides and encapsulated them in PEG-PLA 50 nanoparticles [7]. As advantage in comparison with the mentioned systems, BSA nanoparticles accumulated the oligonucleotide mainly in the nucleus, without addition of other compounds in the formulation.

In this work, two types of albumin carriers were evaluated. The one was obtained by the oligonucleotide adsorption onto pre-formed nanoparticles (PO-NPA). The other consisted in the complexation between BSA and the oligonucleotide prior to the formation of the nanoparticles (PO-NPB). Their main physico-chemical characteristics are shown in Table 1 and more details about these systems as carriers for the phosphodiester oligonucleotide have been previously reported [20]. Their ability to protect PO against enzymatic degradation was modest [20]. Free PO and PO-NPA lost its hybridization capability immediately after incubation with phosphodiesterase at 0.1 mg/ml (Table 3). Only the encapsulation of the oligonucleotide inside the matrix of the nanoparticle was able to maintain the oligonucleotide hybridization capability after enzymatic degradation (Table 3). Also Lambert et al. (2000) [27] found that the encapsulation was much more efficient to protect oligonucleotides against degradation by serum nucleases than the protection obtained by simple adsorption onto CTAB-coated nanospheres [12]. However, either by adsorption or encapsulation, BSA nanoparticles protected oligonucleotides with less efficiency than others systems [28]. It could be explained by the rapid desorption of the oligonucleotides from the nanoparticles surface, promoted by a competition with electrolytes. When the oligonucleotide was encapsulated, the protection against degradation was well correlated with the degradation of BSA nanoparticles over time [20]. A more rapid degradation of BSA nanoparticles (after overnight this carrier was completely degraded) as compared with other types of nanoparticles could explain our unfavorable results.

Since albumin nanoparticles were unable to efficiently protect the oligonucleotide PO against enzymatic degradation (Table 3), but promoted its nuclear accumulation, they were thus used as carriers for the phosphorotioate analog PS in order to combine both, enzymatic stability and major nuclear accumulation. The characteristics of the systems are summarized in Table 1. It has been previously reported that PS oligonucleotides bind much more strongly to proteins (and thus, albumin) than PO oligonucleotides [29], that may partly result from the greater electronegativity of the oxygen in the phosphodiester backbone than the sulfur. This fact would explain why albumin nanoparticles were more efficient carriers for PS that for PO in terms of loading (Table 1) and also exerted a fully protection of the tio-analogue against its partial enzymatic degradation at least overnight (Table 3). However, one of the major problems with phosphorotioate oligonucleotides is their ability to bind in a length- and somewhat sequence-dependent manner to key structural proteins, enzymes, receptors, or growth factors via ionic or hydrophobic bonding [2]. In this way, the delivery of natural phosphodiester oligonucleotides induces less-side effects than modified oligonucleotides and it would be preferable.

Whatever procedure the association of the phosphodiester oligonucleotide with nanoparticles, significantly improved the antiviral activity of the free drug (Fig. 2A). Albumin nanoparticles add the fusogenic properties of the protein to a modest protection of the phosphodiester oligonucleotide against enzymatic degradation only when entrapped in these carriers (PO-NPB). However, in cell cultures, the use of heat inactivated serum prevented any significant influence of differences in enzymatic susceptibility between PO-NPA and PO-NPB. The result was both nanoparticle systems improved the antiviral activity of free oligonucleotide in similar order of magnitude (Fig. 2A). However, in presence of enzymes and, thus, in vivo, a better activity of the oligonucleotide entrapped

into these nanoparticles can be expected. Numerous studies in vitro indicated that oligonucleotide-loaded nanoparticles improved the antisense activity of the oligonucleotide, however, they were non-specific effects due to PIBCA or CTBA [13] because of their positive charge. Our results probed no important antiviral activity for unloaded nanoparticles. Thus, we found an improvement of the oligonucleotide antiviral activity not due to non-specific effect or artefactual results. Finally, the antiviral activity of oligonucleotides was not due to oligonucleotide-induced cytotoxicity because effects on cell viability or proliferation were observed only at high concentrations (data not shown).

On the other side, PS has been described which is internalized by cells in more extension than the unmodified one but also by a endocytic process [30]. More uptake oligonucleotide in addition with more stability against enzymatic degradation of this derivative implied more fractions able to escape from endosomes and to be effective. The effect of albumin in the association of oligonucleotides with cells was evaluated for a phosphodiester one. It could be different for phosphorotioate analogues as far as the binding affinity of the last one for BSA is stronger [29] and in fact, a more significant effect of the protein in decreasing the uptake of modified oligonucleotides as compared with the unmodified one has been previously reported [30]. PS-nanoparticles did not improve the antiviral activity of free PS (Fig. 2B). Thus, it seems that the favorable nuclear accumulation promoted by these carriers would be unable to counteract the observed decrease in the oligonucleotide amount uptake by cells. Moreover, sequence unspecific effects will be probably implied in the increased PS antiviral activity as compared with PO and this effect seems not to be affected by the association of this oligonucleotide to nanoparticles.

In summary, the use of albumin nanoparticles as carriers for oligonucleotides offers a number of advantages. A system so simple negatively charged is able to load oligonucleotides, without requirements of positive compounds [20]. Moreover, albumin nanoparticles promote the nuclear accumulation of the oligonucleotide, without additives destabilizing endosomal membrane. On the other side, their major drawbacks were related to a rapid degradation that limits its ability to protect oligonucleotides against enzymatic degradation and the effect of albumin disturbing the oligonucleotide uptake by an unclarified mechanism.

Albumin-based particulate systems (size 250 nm) are quickly removed from the circulation by opsonization with various serum components followed by phagocytosis by-SMM-rich organs (liver and spleen) [31]. Thus, a phosphodiester oligonucleotide entrapped into BSA nanoparticles will be probably protected enough time to get intact the macrophages, where these vesicles would be able to promote the adequate intracellular compartmentalization to exert its antiviral activity. For the phosphorotioate derivatives, the benefit would be only derived from the ability of these carriers to change the pharmacokinetic profile and target antisense therapy to macrophages.

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References

[1] S. Agrawal, E.R. Kandimalla, Antisense therapeutics: is it as simple as complementary base recognition? Mol. Med. Today 6 (2000) 72–81.

[2] I. Lebedeva, C.A. Stein, Antisense oligonucleotides: promise and reality, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 403–419.

[3] C. Beltinger, H. Saragovi, R. Smith, L. LeSauteur, N. Shah, L. De Dionisio, L. Christensen, A. Raible, L. Jarett, A. Gewirtz, Binding, uptake, and intracellular trafficking of phosphorothioate-modified oligodeoxynucleotides, J. Clin. Invest. 95 (1995) 1814–1823.

[4] J. Hughes, A. Astriab, H. Yoo, S. Alahari, E. Liang, D. Sergueev, B.R. Shaw, R.L. Juliano, In vitro transport and delivery of antisense oligonucleotides, Methods Enzymol. 313 (2000) 342–358.

[5] R.L. Juliano, S. Alahari, H. Yoo, R. Kole, M. Cho, Antisense pharmacodynamics: critical issues in the transport and delivery of antisense oligonucleotides, Pharm. Res. 16 (1999) 494–502.

[6] D.D. Stuart, T.M. Allen, A new liposomal formulation for antisense oligodeoxynucleotides with small size, high incorporation efficiency and good stability, Biochim. Biophys. Acta 1463 (2000) 219–229.
[7] C. Emile, D. Bazile, F. Herman, C. Hélène, M. Veillard, Encapsulation of oligonucleotides in stealth Me.PEG-PLA50 nanoparticles by complexation with structured oligopeptides, Drug Deliv. 3 (1996) 187–195.
[8] H. Fritz, M. Maier, E. Bayer, Cationic polystyrene nanoparticles: preparation and characterization of a model drug carrier system for antisense oligonucleotides, J. Colloid Interf. Sci. 195 (1997) 272–288.
[9] K.A. Janes, P. Calvo, M.J. Alonso, Polysaccharide colloidal particles as delivery systems for macromolecules, Adv. Drug Del. Rev. 47 (2001) 83–97.
[10] I. Aynié, C. Vauthier, H. Chacun, E. Fattal, P. Couvreur, Spongelike alginate nanoparticles as a new potential system for the delivery of antisense oligonucleotides, Antisense Nucleic Acid Drug Dev. 9 (1999) 301–312.
[11] G. Schwab, C. Chavany, I. Duroux, G. Goubin, J. Lebeau, C. Hélène, T. Saison-Behmoaras, Antisense oligonucleotides adsorbed to polyalkylcyanoacrylate nanoparticles specifically inhibit mutated Ha-ras-mediated cell proliferation and tumorigenicity in nude mice, Proc. Natl. Acad. Sci. USA 91 (1994) 10460–10464.
[12] C. Chavany, T. Saison-Behmoaras, T. Le Doan, F. Puisieux, P. Couvreur, C. Hélène, Adsorption of oligonucleotides onto polyisohexylcyanoacrylate nanoparticles protects them against nucleases and increases their cellular uptake, Pharm. Res. 11 (1994) 1370–1380.
[13] G. Lambert, E. Fattal, A. Brehier, J. Feger, P. Couvreur, Effect of polyisobutylcyanoacrylate nanoparticles and lipofectin loaded with oligonucleotides on cell viability and PKC alpha neoshyntesis in HepG2 cells, Biochimie 80 (1998) 969–976.
[14] C. Chavany, T. Le Doan, P. Couvreur, F. Puisieux, C. Hélène, Polyalkylcyanoacrylate nanoparticles as polymeric carriers for antisense oligonucleotides, Pharm. Res. 9 (1992) 441–449.
[15] G. Godard, A.S. Boutorine, T. Saison-Behmoaras, C. Hélène, Antisense effects of cholesterol-oligodeoxynucleotide conjugates associated with poly(alkylcyanoacrylate) nanoparticles, Eur. J. Biochem. 232 (1995) 404–410.
[16] D. Bazile, C. Prud'homme, M. Bassoullet, M. Marlard, G. Spenlehauer, M. Veillard, Stealth Me.PEG-PLA nanoparticles avoid uptake by the mononuclear phagocytes system, J. Pharm. Sci. 84 (1995) 493–498.
[17] D.C. Drummond, M. Zignani, J.C. Leroux, Current status of pH-sensitive liposomes in drug delivery, Prog. Lipid Res. 39 (2000) 409–460.
[18] M. Berton, E. Allemann, C.A. Stein, R. Gurny, Highly loaded nanoparticulate carrier using an hydrophobic antisense oligonucleotide complex, Eur. J. Pharm. Sci. 9 (1999) 163–170.
[19] S. General, A.F. Thunemann, pH-sensitive nanoparticles of poly(amino acid) dodecanoate complexes, Int. J. Pharm. 230 (2001) 11–24.
[20] A. Arnedo, S. Espuelas, M.J. Renedo, J.M. Irache, Albumin nanoparticles as carriers for a phosphodiester oligonucleotide, Int. J. Pharm. 244 (1–2) (2002) 59–72.
[21] A. Arnedo, J.M. Irache, G. González Gaitano, M. Valgañón, S. Espuelas, Bovine serum albumin modified the intracellular distribution and improved the antiviral activity of an oligonucleotide, J. Drug Targeting 11 (2003) 197–204.
[22] Y. Sato, K. Kaneko, K. Mikami, M. Mizugaki, Y. Suzuki, Isolation of bovine serum albumin fragment P-9 and P-9 mediated fusion of small unilamellar vesicles, Biol. Pharm. Bull. 22 (1999) 1360–1365.
[23] S. Simoes, V. Slepushkin, P. Pires, R. Gaspar, M.C. Pedroso de Lima, N. Düzgünes, Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum, Biochim. Biophys. Acta 1463 (2000) 459–469.
[24] R.F. Azad, V.B. Driver, K. Tanaka, R.M. Crooke, K.P. Anderson, Antiviral activity of a phosphorothioate oligonucleotide complementary to RNA of the human cytomegalovirus major immediate–early region, Antimicrob. Agents Chemother. 37 (1993) 1945–1954.
[25] K.J. Lewis, W.J. Irwin, S. Akhtar, Development of a sustained-release biodegradable polymer delivery system for site-specific delivery of oligonucleotides: characterization of P(LA-GA) copolymer microspheres in vitro, J. Drug Targeting 5 (1998) 291–302.
[26] A.J. Hudson, W. Lee, J. Porter, J. Akhtar, R. Duncan, S. Akhtar, Stability of antisense oligonucleotides during incubation with a mixture of isolated lysosomal enzymes, Int. J. Pharm. 133 (1996) 257–263.
[27] G. Lambert, E. Fattal, H. Pinto-Alphandary, A. Gulik, P. Couvreur, Polyisobutylcyanoacrylate nanocapsules containing an aqueous core as a novel colloidal carrier for the delivery of oligonucleotides, Pharm. Res. 17 (2000) 707–714.
[28] H.P. Zobel, J. Kreuter, D. Werner, C.R. Noe, G. Kumel, A. Zimmer, Cationic polyhexylcyanoacrylate nanoparticles as carriers for antisense oligonucleotides, Antisense Nucleic Acid Drug Dev. 7 (1997) 483–493.
[29] S.K. Srinivasan, H.K. Tewary, P.L. Iversen, Characterization of binding sites, extent of binding, and drug interactions of oligonucleotides with albumin, Antisense Res. Dev. 5 (1995) 131–139.
[30] T. Kanamaru, T. Takagi, Y. Takakura, M. Hashida, Biological effects and cellular uptake of c-myc antisense oligonucleotides and their cationic liposome complexes, J. Drug Targeting 5 (1998) 235–246.
[31] S.P. Vyas, V. Sihorkar, Endogenus carriers and ligands in non-immunogenic site-specific drug delivery, Adv. Drug Del. Rev. 43 (2000) 101–164.