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Albumin nanoparticles as carriers for a phosphodiester oligonucleotide

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Abstract

The goal of this study was the evaluation of albumin nanoparticles as drug delivery systems for antisense oligonucleotides. Bovine serum albumin (BSA) nanoparticles were prepared by a coacervation process. A phosphodiester oligonucleotide was either incorporated into the matrix of the particles by incubation with the albumin prior the coacervation process or adsorbed onto the pre-formed nanoparticles. Incorporated and/or adsorbed oligonucleotide was estimated by capillary electrophoresis and fluorescence spectroscopy. The adsorbed amount of oligonucleotide was dramatically dependent on the pH of the medium. Desorption of the oligonucleotide was also affected by the pH and ionic strength of the medium. This indicated that electrostatic forces play a major role in the interaction between the oligonucleotide and the nanoparticles. When the oligonucleotide was incubated with the albumin prior to nanoparticle formation, the profile of release confirmed that a fraction was incorporated into the matrix and its release was controlled by the albumin degradation. The hybridisation capability of the oligonucleotide in both nanoparticle formulations was retained. However, only the oligonucleotide incorporated into the nanoparticle matrix was protected against enzymatic degradation.

Keywords: Nanoparticles; Oligonucleotide; Albumin; Adsorption; Degradation; Stability

1. Introduction

Antisense oligonucleotides are synthetic fragments of ribo- or deoxyribonucleic acids that recognise and bind specifically to the complementary sequence of a gene or its messenger RNA. Their hybridisation is thought to interfere with processing, transport and/or translation as well as to elicit degradation of the target RNA, leading to the inhibition of the target gene expression (Lebedeva et al., 2000).

A number of these molecules are of potential interest for clinical applications that can exploit their antiviral properties. In this context, different antisense oligonucleotides have been proposed as therapeutics against human immunodeficiency virus (Zamecnik et al., 1986), herpes simplex virus (Smith et al., 1986), influenza virus (Abe et al., 2001), Rous sarcoma virus (Stephenson and Zamecnik, 1978) and vesicular stomatitis viruses (Lemaitre et al., 1987).

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However, the efficacy of oligonucleotide-based therapies is usually limited by the instability of these molecules in biological fluids. In addition, their poor capacity to diffuse through the biological membranes and their inadequate cellular compartmentalisation to reach its target site into the cell may dramatically limit the efficiency of these new drugs (Berton et al., 1999). In order to decrease their degradation rate in biological fluids, a common approach may be the introduction of some chemical modifications in their structure. One of the most frequent modifications is the change of a non-bridging O-atom of the phosphodiester backbone by a sulfur atom (Lebedeva et al., 2000). These derivatives, called phosphorothioates, and many other oligomer classes that are experimentally employed, possess a high stability against degradation induced by nucleases. However, these oligonucleotides suffer from two major inconveniences. The former is the loss, in many cases, of their antisense activity (Lambert et al., 2001a). The latter is that their capacity to penetrate across membranes remains poor due to their polyanionic structure (Berton et al., 1999). In order to overcome these drawbacks and to increase the cellular uptake of oligonucleotides, a number of technological strategies have been assayed including the use of colloidal drug delivery systems.

In this context, a number of lipid vesicles have been proposed, including cationic liposomes (Wilhelm et al., 1995), virosomes (Kaneda, 2000), pH sensitive- (de Oliveira et al., 2000), fusogenic- (Fresta et al., 1998) and immuno-liposomes (Leserman et al., 1980). In spite of some advantages offered by these carriers, their main inconveniences are related to their technical complexity and their instability in the presence of serum (Lebedeva et al., 2000).

Another possibility to both protect natural unmodified phosphodiester oligonucleotides (PO) and to increase its cellular uptake may be the use of biodegradable nanoparticles. An electrostatic adsorption of PO onto nanospheres from poly(alkylcyanoacrylate) derivatives (Chavany et al., 1992; Lambert et al., 1998; Zobel et al., 2000) was possible by the coating of these carriers with positively charged compounds such as cetyltrimethylammonium bromide (Chavany et al., 1992), DEAE-dextran (Zobel et al., 2000) or cationic lipids (Lambert et al., 1998). These systems appear to increase the amount of PO associated to cells and, thus, their efficacy. More recently, formulations able to encapsulate PO, rather than a simple electrostatic adsorption have also been developed, such as poly(isobutylcyanoacrylate) nanocapsules (Lambert et al., 2001b) and poly(lactic acid) nanoparticles (Berton et al., 1999). As compared with the simple adsorption, the PO encapsulation appeared to improve its protection against enzymatic degradation. However, these encapsulating-carriers are difficult to obtain and require the use of aggressive conditions that compromise the PO stability. In this sense, the electrostatic complexation represents a considerable advantage over encapsulation because it allows the use of pre-formed nanoparticulate formulations.

Another system suitable for the delivery of antisense oligonucleotides could be the use of nanoparticles from proteins. Albumin nanoparticles have been extensively studied in previous works as suitable for drug delivery (Lin et al., 1993; Merodio et al., 2001) since they are biodegradable (Morimoto and Fujimoto, 1985), non-toxic (Rhodes et al., 1969) and non antigenic (Arshadi, 1990). Because of their defined primary structure, and high content of charged amino acids (i.e. lysine), the albumin-based nanoparticles could allow the electrostatic adsorption of positively or negatively charged molecules without the requirements of other compounds. In addition, protein nanoparticles can be easily prepared under soft conditions, by coacervation or controlled desolvation processes, and incorporate several types of molecules (Lin et al., 1993). It is also well known that serum albumin may bind spontaneously acidic compounds such as oligonucleotides (Geselowitz and Neckers, 1995). So far, the fusogenic properties of serum albumin at low pH have been previously described (Sato et al., 1999). The fact could modify the inadequate intracellular distribution of oligonucleotides and improves their efficacy.

The present work evaluates the feasibility of bovine albumin nanoparticles to carry a 21-mer

phosphodiester oligonucleotide complementary to the RNA of the major IE transcriptional unit of human cytomegalovirus. In fact, its phosphorothioate modification, called ISIS 2922, shows a potent antiviral activity against human cytomegalovirus and has been recently approved by the FDA. We studied the ability of albumin nanoparticles to carry the oligonucleotide adsorbed onto the surface or entrapped into the matrix. Also the protection against enzymatic degradation exerted by both systems was assayed.

2. Materials and methods

2.1. Materials

21-mer oligonucleotide (PO) sequence 5′-GCGTTTGCTCTTCTTCTTGCG-, both, unlabelled and labelled at the 5′-end with fluorescein isothiocyanate (FITC-PO) were provided by Pharmacia Biotech (Cambridge, UK). Bovine serum albumin (BSA, fraction V), methanol, chlorhydric acid, potassium dihydrogen phosphate and monoacid sodium phosphate decahydrated were purchased from Merck (Darmstadt, Germany). Glutaraldehyde (grade II, 25%), sodium hydroxide, sodium chloride, copper (II) sulfate, bicinchoninic acid solution and proteinase K were obtained from SIGMA (Madrid, Spain). Sodium borate was from PANREAC (Barcelona, Spain) and ethanol absolute from PROLABO (Fontenay, France). SYBR® Green I nucleic acid gel stain were provided by Molecular Probes (Eugene, OR).

2.2. Preparation of PO loaded albumin nanoparticles

Nanoparticles were prepared by a coacervation process and cross-linkage with glutaraldehyde following two different procedures. For this purpose, PO was incubated with either the pre-formed albumin carriers (PO-NPA) or an albumin solution prior the formation of nanoparticles (PO-NPB).

Briefly, PO-NPA were obtained by the addition of ethanol dropwise (ethanol:water relation 2:1) to an aqueous solution of BSA (2% w/v), adjusted to a pH 5.5 with HCl 0.1N. Coacervates thus obtained were then hardened with different glutaraldehyde concentrations (from 0.06 to 12.5 μg/mg) 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 17000 r.p.m. for 30 min (Sorvall RC-plus, rotor SS-34, Connecticut, USA) to eliminate free albumin and the excess of the cross-linking agent. The supernatants were removed and the pellets resuspended in an aqueous solution (pH from 3 to 9) containing variable amounts of PO (ranging from 2 to 40 μg/mg nanoparticle) and incubated for 2 h. Then, PO-NPA were separated from the free drug by centrifugation (17000 r.p.m./10 min) at 4 °C.

For PO-NPB, a variable amount of the oligonucleotide (ranging from 2 to 20 μg/mg nanoparticle) was firstly incubated with the albumin aqueous solution (2% w/v; pH 5.5). Then, this aqueous phase was desolvated with ethanol dropwise (ethanol:water relation 2:1). Coacervates so formed were hardened with different glutaraldehyde concentrations (from 0.06 to 12.5 μg/mg) for 2 h and the resulting nanoparticles were purified by centrifugation as described above.

In all cases, the purified nanoparticles by centrifugation were resuspended and dispersed in either a phosphate or a citrate buffer.

2.3. Size and zeta potential determinations

The size and zeta potential of loaded and unloaded albumin nanoparticles were determined in a Zetamaster analyser system (Malvern Instruments, UK). The samples were diluted with distilled water and measured at room temperature with a scattering angle of 90°. The size and shape of the nanoparticles were also examined by scanning electron microscopy (SEM) in a JEOL JSM-6400 scanning microscope (JEOL LTD, Japan).

2.4. Nanoparticle yield

The amount of protein transformed into nanoparticles was determined by a standard BCA

protein assay. The nanoparticulate pellet, obtained after centrifugation, was digested with NaOH 0.1M under magnetic stirring for 2 h at room temperature. Then, the resulting solutions were analysed in a spectrophotometer at 562 nm. In all cases, the resulted absorbance was compared with the data obtained after digestion of a control albumin solution. A rectilinear calibration curve from 10 to 100 μg/ml ($r^2 > 0.976$) was performed using a control albumin solution in NaOH 0.1 M.

2.5. PO analysis

The amount of PO loaded into albumin particles was calculated as the difference between the total amount of the initial antisense oligonucleotide added and the amount of PO determined in the supernatants obtained during the purification step of the nanoparticles by centrifugation. Determinations were carried out by capillary zone electrophoresis (Arnedo et al., 2000) and spectrofluorimetry.

Electrophoresis (CZE) was performed by using a HPCE apparatus (Hewlett Packard, Waldbronn, Germany) equipped with an automatic sampling system and a diode-array detector, set at 270 nm (reference 325 nm). For the separations, a fused-silica capillary of 48.5 cm (40 cm to the detector) × 50 μm I.D., filled with borate buffer (12.5 mM, pH 9.5) was used. Potential 30 kV was applied and the capillary temperature was 30 °C. For analysis, samples were spiked with 25 μl of the internal standard (paracetamol, 0.05 mg/ml), diluted to 1 ml with double-distilled water and injected by pressure at 50 mbar for 10 s. The limit of quantification was calculated to be around 1.27 μg oligonucleotide and the precision and accuracy of the method did not exceed 7%.

On the other hand, the amount of FITC-PO was assessed in the supernatant by fluorimetry in a Cytofluor™ 2350 (Fluorescence Measurement system, Millipore). For this purpose, the supernantants obtained during the purification steps of the nanoparticle production were diluted in NaOH 1 M and directly measured in the fluorimeter at 530 nm.

The drug loading was calculated as the ratio between the amount of drug in nanoparticles (expressed in μg) and the yield of the preparative process of albumin nanoparticles (expressed in mg). In addition, the drug entrapment efficiency was defined as the percentage of PO loaded relating to the initial amount of antisense oligonucleotide.

2.6. 'In vitro' release studies

A total of 15 μg PO-loaded albumin nanoparticles were dispersed in 1 ml release media in eppendorf tubes. The release media used here were either PBS pH 7.4 (0.05, 0.15 or 1 M) or citrate buffer 0.19 M (pH 3, pH 5, pH 6.5 or pH 7.4). Then, eppendorf tubes were placed in a shaking bath at 37 ± 1 °C with a constant agitation of 60 strokes/min (Unitronic 320 OR, Selecta, Madrid, Spain). At predetermined intervals, the samples were centrifuged. In the supernatants, the amount of oligonucleotide released was determined by CZE or fluorimetry. Meanwhile, in the bottoms, the amount of undegraded nanoparticles was determined by the standard BCA protein assay as described above. The amount of degraded nanoparticles were obtained as difference between the initial amount of nanoparticles and the undegraded amount of nanoparticles and expressed as percentage. The analysis was performed by triplicate for each sample.

2.7. Hybridisation capability of loaded PO

The hybridisation capability of the 21-mer oligonucleotide loaded in albumin nanoparticles was determined using a fluorescence temperature cycler (Lightcycler, Roche Diagnostics GmbH, Germany). First of all, oligonucleotides were extracted from nanoparticles by degradation of the macromolecule matrix. For this purpose, 1 ml suspension PO-nanoparticles containing 20 μg PO were degraded by incubation with 0.1 mg/ml proteinase K at 50 °C for 2 h. Then, samples (20 ng aliquots antisense oligonucleotide) were incubated with its complementary sense oligonucleotide in a 1:1 ratio in the presence of 1 μl SYBR® Green (1:1000) diluted in 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 bound to the duplex double stranded. The fluorescence signal is proportional to the hybridisation 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 visualisation of the T_m (temperature at which 50% of the originally added PO is still in a duplex; Lewis et al., 1998, melting peaks were derived from the initial melting curves (fluorescence [F] versus temperature [T] by plotting the negative derivative of fluorescence over temperature versus temperature ($[-dF/dT]$ versus T).

2.8. Enzymatic stability in presence of phosphodiesterase

Free oligonucleotide (PO), PO-NPA, or PO-NPB were incubated with snake venom phosphodiesterase (0.1 mg/ml) for 5 min, 60 min or overnight. Degradation experiments were carried out in a shaking bath at 37 °C ± 1 °C, with a constant agitation of 60 strokes/min (Unitronic 320 OR, Selecta. Madrid, Spain) in buffer 100 mM Tris–HCl (pH 8.9), 100 mM NaCl and 14 mM $MgCl_2$. Heating at 80 °C for 5 min stopped the degradation. Then, samples were incubated with 0.1 mg/ml of proteinase K at 50 °C for 2 h to degrade the albumin and the hybridisation capability of PO was determined as described above.

3. Results

3.1. PO analysis

The amount of the PO loaded nanoparticles was estimated by either capillary electrophoresis (CZE) or spectrofluorimetry. Fig. 1 displays a typical electropherogram from the supernatants obtained during the purification step. The oligonucleotide corresponding peak appeared at 4.2 min. No interference with albumin and other components used during the preparation of nanoparticles were observed. On the other hand, when the fluorescently labelled PO was used, the antisense molecule was quantified by spectrofluorimetry. Both techniques (CZE and fluorimetry) provided similar results. In fact, a fairly lineal relationship ($r^2 = 0.9916$) between drug loading results obtained by capillary electrophoresis and those determined by fluorometry was found (data no shown).

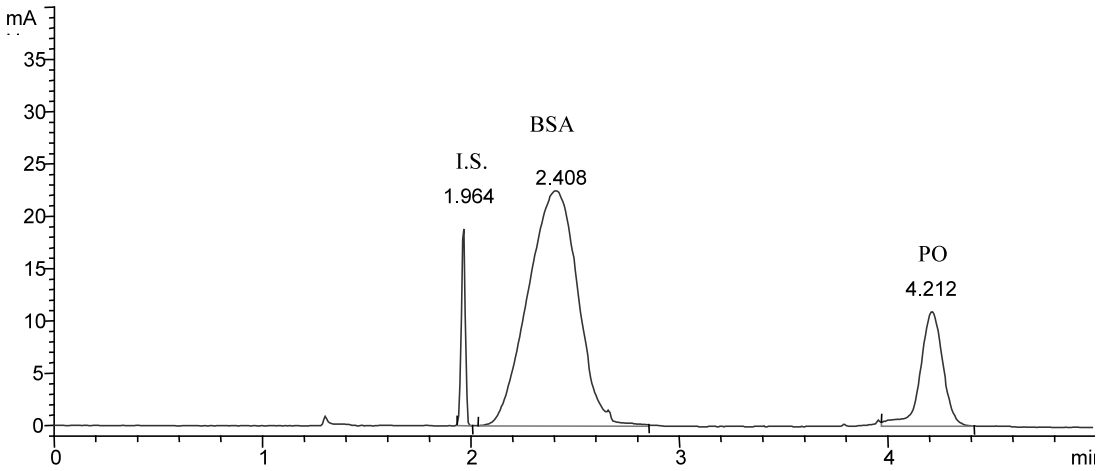

Fig. 1. Electropherogram resulting from the analysis of the supernatant obtained during the purification step of oligonucleotide loaded nanoparticles. I.S., internal standard (paracetamol); BSA, serum albumin bovine; PO, oligonucleotide.

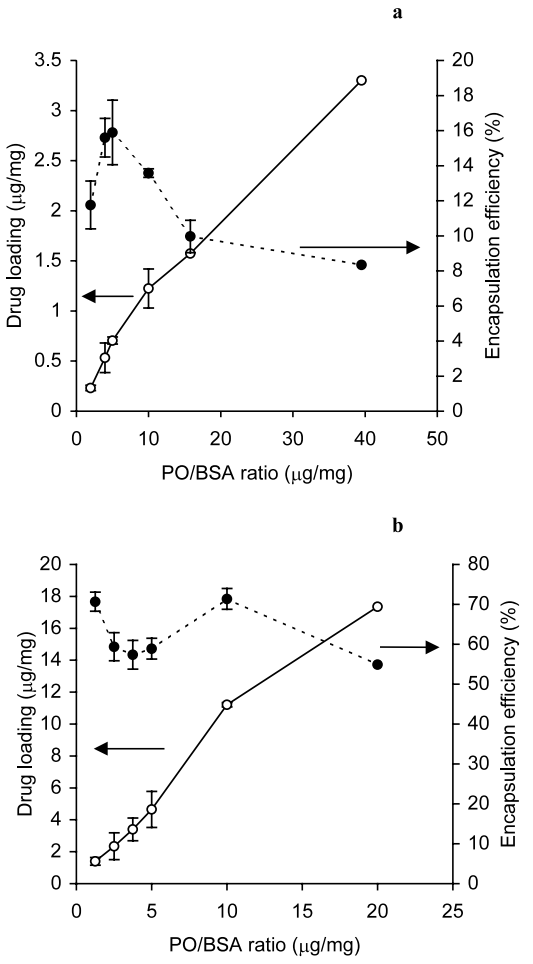

Fig. 2. Influence of the PO/albumin ratio on the drug loading (μg PO/mg nanoparticle) and the entrapment efficiency (expressed in %). (a) PO-NPA, nanoparticles incubated for 2 h with the drug. (b) PO-NPB, nanoparticles prepared after 30 min of incubation between the albumin aqueous solution and the drug. Error bars represent calculations of standard error on the basis of triplicate determinations. Nanoparticles were prepared with a glutaraldehyde concentration of 1.56 μg/mg.

3.2. Optimisation of nanoparticle preparation

3.2.1. Influence of the bulk PO

Fig. 2a and b show the capacity of the two different procedures for loading the oligonucleotide as a function of the drug/initial protein ratio (PO/albumin ratio). The glutaraldehyde concentration was fixed to 1.56 μg/mg. When the PO was incubated with the empty nanoparticles (PO-NPA), the payload increased with the concentration of the oligonucleotide (see Fig. 2a). For PO-NPB, this increase on the drug loading was significantly higher than for PO-NPA, mainly at oligonucleotide/protein ratios higher than 5 μg PO/albumin. With respect to the encapsulation efficiency, near 70% were obtained for PO-NPB, while PO-NPA only showed a maximum close to 16%. The influence of the incubation time between the PO and either the nanoparticles or the free protein on the drug loading was also studied. However, under the experimental conditions tested (incubation time between 0.25 and 24 h), no significant differences in the payload were found ($P < 0.05$, data not shown).

3.2.2. Influence of pH on the PO loading to pre-formed nanoparticles

In order to provide information about the nature of interaction between the surface of albumin nanoparticles and oligonucleotides, adsorption studies were subsequently carried out in mediums with different pH conditions. Table 1 summarises the influence of the pH conditions, in which the incubation between the oligonucleotide and the pre-formed nanoparticles was carried out, on the drug loading of the resulted carriers. At pH conditions ranging from 4 to 9, the unloaded carriers displayed a negative zeta potential and their capacity to load the antisense oligonucleotide was very low. On the contrary, at pH 3, the zeta potential of albumin nanoparticles was positive and, therefore, their capacity to load polyanions (i.e. antisense oligonucleotides) significantly increased.

Table 1
Influence of the pH on the zeta potential of empty albumin nanoparticles and the capacity of these carriers to load the 21-mer antisense (PO-NPA)

pH	Zeta Potential (mV)	Drug loading (μg/mg)
3	$+12.3 \pm 0.4$	3.00 ± 0.33
4	-23.3 ± 0.6	0.65 ± 0.16
6	-24.6 ± 0.4	0.60 ± 0.22
7.4	-24.2 ± 0.1	0.75 ± 0.11
9	-27.1 ± 0.3	0.47 ± 0.09

Experimental conditions: PO/BSA ratio of 5 μg/mg. Data are expressed as the mean ± S.D. ($n = 3$).

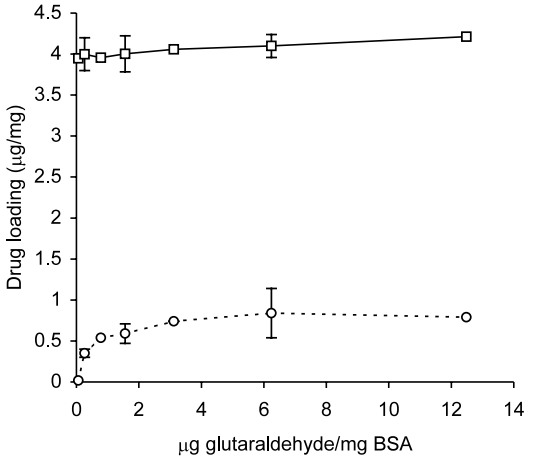

Fig. 3. Influence of the cross-linkage on the oligonucleotide loading in PO-NPA and PO-NPB at a PO/albumin ratio of 5 μg PO/mg BSA (-○-, PO-NPA; -□-, PO-NPB).

3.2.3. Influence of the cross-linking agent on the PO loading

Fig. 3 shows the influence of the cross-linking agent/protein ratio on the oligonucleotide loading in albumin nanoparticles. The medium was water acidified with HCl (pH around 5.5). For PO-NPB, the cross-linkage of albumin nanoparticles did not significantly influence the PO loading. Meanwhile, increasing amounts of glutaraldehyde to harden the NPA increased the payload of the oligonucleotide till a maximum of around 3 μg glutaraldehyde/mg protein (Fig. 3). However, the crosslinkage of the nanoparticles was optimised with 1.56 μg/mg, since higher aldehyde amounts resulted in a significant polydispersity increase (data not shown).

3.3. Characterisation of the 21-mer PO-loaded nanoparticle formulations

Table 2 compares the main physicochemical properties of both types of formulations, prepared with an oligonucleotide/albumin ratio of 5 μg/mg. At this ratio, PO-NPA and PO-NPB formulations were characterised by both a slightly lower size and a slightly higher yield than unloaded albumin nanoparticles. Concerning the zeta potential, the values were always negative, in the range -20 ± 5 mV. In addition, the drug loading was around 6-fold lower when the oligonucleotide was incubated with pre-formed nanoparticles (PO-NPA) that when it was incubated with the native protein prior the formation of nanoparticles (PO-NPB). Thus, the encapsulation efficiency was calculated to be close to 16%, however, for PO-NPB, this efficacy raised to 59%. Finally, the SEM microphotograph (see Fig. 4) clearly showed PO-NPB spherical-shaped, homogeneous and not aggregated, whose size was similar to the value obtained by photon correlation spectroscopy.

3.4. 'In vitro' release studies

PO-NPA and PO-NPB formulations containing 15 μg oligonucleotide were tested for in vitro release at 37 ± 1 °C. Fig. 5 displays the plot of the data expressed as the cumulative amount of PO released from the two albumin nanoparticle formulations as a function of time. The two formulations displayed different release profiles. For the PO-NPA formulation, a complete and instantaneous release of almost the whole adsorbed oligonucleotide was observed.

Table 2
Influence of the preparative model on the physicochemical characteristics of the oligonucleotide-loaded nanoparticles

	Size (nm)	Yield (%)	Zeta Potential (mV)	Drug loading (μg PO/mg NP)	Entrapment efficiency (%)
Unloaded NP	258 ± 1	56.6 ± 1.7	-24.8 ± 3.0	–	–
PO-NPA	248 ± 5	57.8 ± 1.8	-24.1 ± 0.3	0.75 ± 0.11	15.94 ± 1.81
PO-NPB	246 ± 6	60.5 ± 0.7	-21.6 ± 0.9	4.67 ± 0.27	58.94 ± 2.63

Experimental conditions: PO/BSA ratio of 5 μg/mg. Data are expressed as the mean ± S.D. ($n = 3$).

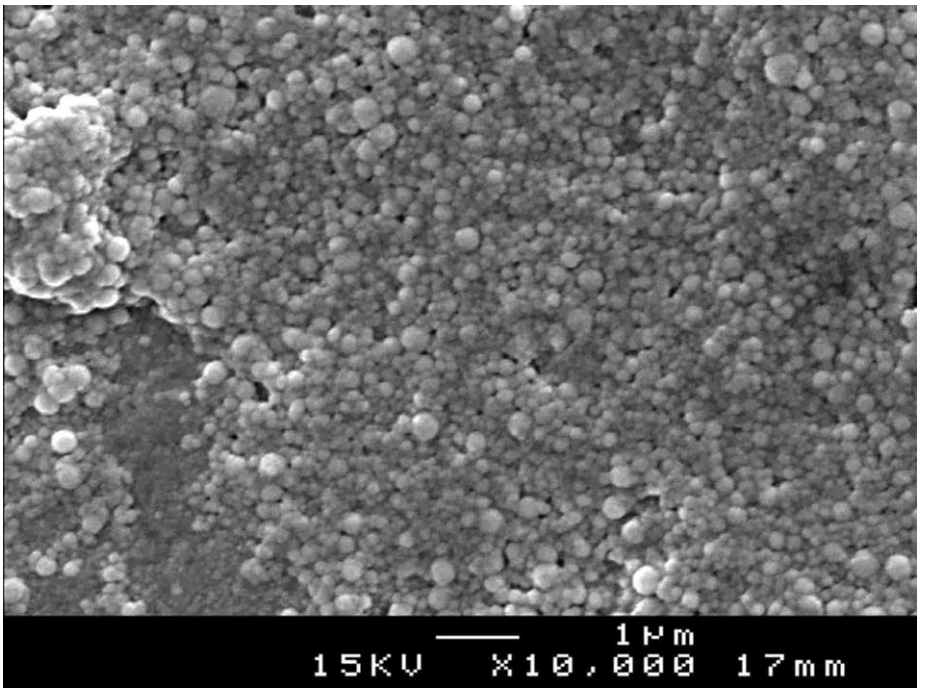

Fig. 4. Scanning electron micrograph of PO-NPB with an average diameter of about 250 nm.

For PO-NPB, the oligonucleotide was released in a biphasic way characterised by an initial burst effect in which around 37% was released, followed by a continuous and slower release with a maximum cumulative value of around 80% of the loaded oligonucleotide after 5 h.

In order to gain insights into factors controlling the release of the oligonucleotide from carriers, its release profile was studied in mediums with variable ionic strengths and different pH. These results for PO-NPA and PO-NPB are summarised in Table 3 and Fig. 6 respectively. As shown in Table 3, desorption of the 21-mer oligonucleotide from the surface of PO-NPA can be induced by increasing either the pH or the ionic strength of the dispersion medium. For PO-NPB, the ionic strength (Fig. 6a) and the pH conditions (Fig. 6b) markedly influenced the burst effect. By increasing the ionic strength of the release medium (Fig. 6a), the burst effect dramatically increased: the initial release raised from 35% of the loaded oligonucleotide, when disposed in PBS 0.005 M, to 60% when the release medium was PBS 1 M. The amount of cumulative oligonucleotide release over 18 h was about 70% at 0.005 M, 75% at 0.15 M and 95% at 1 M.

Fig. 6b shows the influence of pH in PO-NPB release profile. The amount of PO released from albumin nanoparticles increased by raising the pH

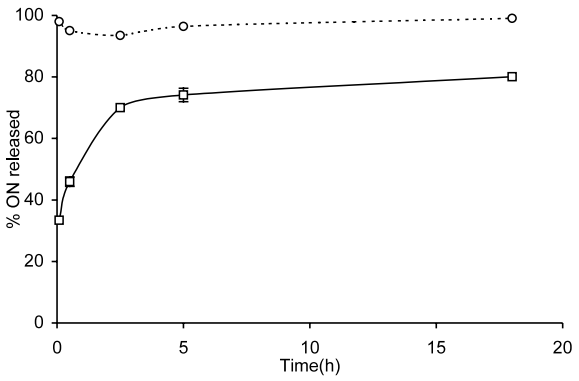

Fig. 5. Release profiles of oligonucleotide from albumin nanoparticles (-○-, PO-NPA; -□-, PO-NPB) in PBS pH 7.4 (0.15 M) at 37 °C. Nanoparticles were prepared with a oligonucleotide/BSA ratio of 5 μg/mg.

Table 3
Influence of the pH and ionic strength on the release profile of the oligonucleotide from PO-NPA

Release media	% PO released
Effect of pH (Ionic strength = 0.19 M)	
pH 5.0	11.96 ± 0.25
pH 6.5	31.66 ± 2.02
pH 7.4	95.47 ± 1.72
Effect of ionic strength (pH 7.4)	
0.05 M	13.88 ± 0.15
0.15 M	48.07 ± 2.72
1.00 M	90.69 ± 1.54

Experimental conditions: PO/BSA ratio of 5 μg/mg; phosphate buffer (pH 7.4) at 0.05, 0.15 and 1 M and citrate buffer (0.19 M) at pH 5, 6.5 and 7.4. Data are expressed as the mean ± S.D. ($n = 3$).

of the release medium over the range between pH 5 and 7.4. In acidic conditions (pH 5 and pH 6.5) the burst release was quite low, around 15 and 30% of the loaded oligonucleotide, respectively. However, at neutral pH, the burst effect represented at least 50% of the loaded drug. On the contrary, strong acidic conditions (pH 3) showed around 80% of burst effect.

In different pH media, a fairly lineal relationship ($r^2 > 0.936$) between the cumulative % of PO released versus time and the nanoparticles degradation in the course of time was found (Fig. 7). This fact indicated that the effect of different media in albumin degradation was the main factor controlling the release of PO incorporated into the nanoparticles. In that sense, the preparation of albumin nanoparticles with a higher amount of glutaraldehyde (3.12 μg glutaraldehyde/mg albumin) slighly decreased the rate of carrier degradation and, thus, the oligonucleotide release. These carriers displayed a burst effect near 24% versus around 47% with 1.56 μg glutaraldehyde/mg albumin (data not shown).

3.5. Hybridisation capability of loaded PO: determination of the melting curve

To ascertain if the process of nanoparticle preparation induced oligonucleotide cleavage, the hybridisation capability of the oligonucleotide loaded to albumin nanoparticles was examined. The melting curves can differentiate the degraded from undegraded antisense oligonucleotides, because the T_m of degraded products will be lower. Results obtained demonstrated that the 21-mer oligonucleotide (T_m for PO of about T_m: 66.33) remained intact when incorporated in either empty nanoparticles or during the preparative process of albumin nanoparticles by coacervation in both PO-NPA (T_m: 67.45) and PO-NPB (T_m: 67.08) (Fig. 8).

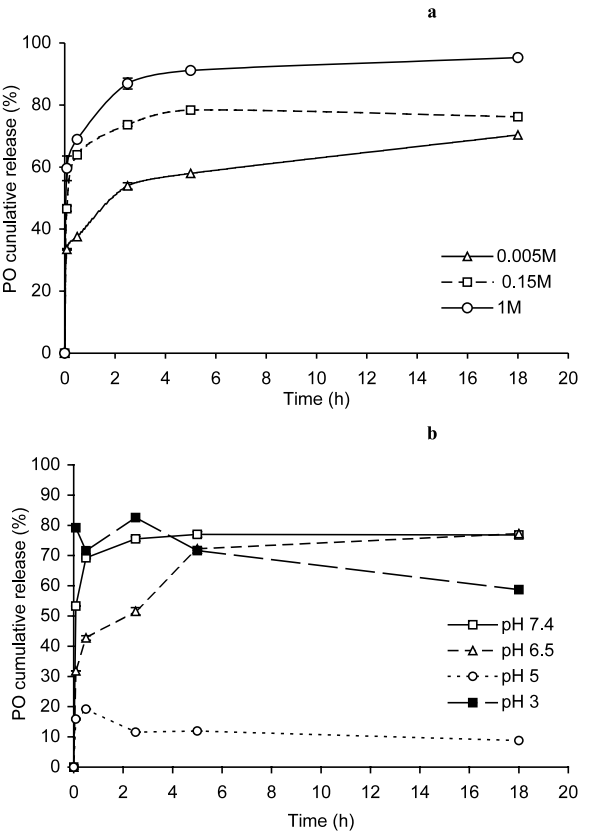

Fig. 6. Influence of the ionic strength (a) and pH conditions (b) on the release profile of the 21-mer oligonucleotide from PO-NPB. Experimental conditions: PO/BSA ratio of 5 μg/mg. Release mediums: (a) phosphate buffers (pH 7.4) at 0.005 M (△), 0.15 M (□), and 1 M (○). (b) Citrate buffers (0.19 M) at pH 7.4 (□), 6.5 (△), 5 (○) and 3 (■).

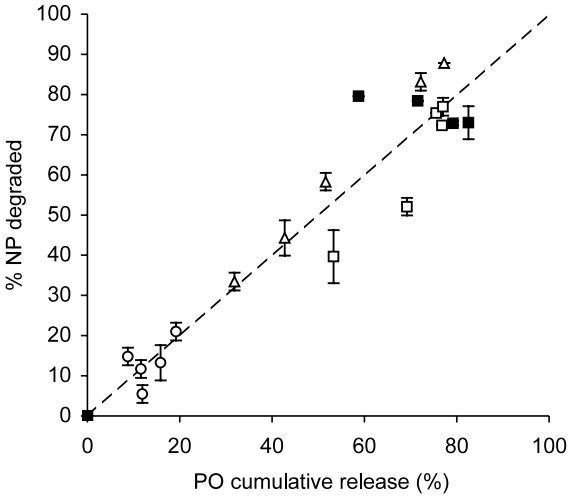

Fig. 7. Comparison of in vitro oligonucleotide released (%) in citrate buffers with different pH conditions (0.19 M), and nanoparticle degradation (%). Bisection line, theoretical curve [(□) citrate buffer 0.19 M, pH 7.4, (△) citrate buffer 0.19 M, pH 6.5 (○), citrate buffer 0.19 M, pH 5 and (■) citrate buffer 0.19 M, pH 3]. Error bars represent calculations of standard error on the basis of triplicate determinations. Experimental conditions: PO/BSA ratio of 5 μg/mg, glutaraldehyde/bulk albumin 1.56 μg/mg.

3.6. Enzymatic stability in presence of phosphodiesterase

We evaluated the ability of albumin nanoparticles to protect oligonucleotides against enzymatic degradation. The enzyme used was a 3′-exonuclease, that hydrolyses the phosphate linkage of oligonucleotides from their 3′-end. The hybridisation capability of oligonucleotides after enzymatic degradation (as T_m values) is shown in Table 4. Both, free PO and PO adsorbed onto nanoparticles were degraded in the first five minutes. Nevertheless, PO-NPB when incubated with the phosphodiesterase, PO–NPB was able to protect the loaded oligonucleotide for 60 min, when incubated with the phosphodiesterase.

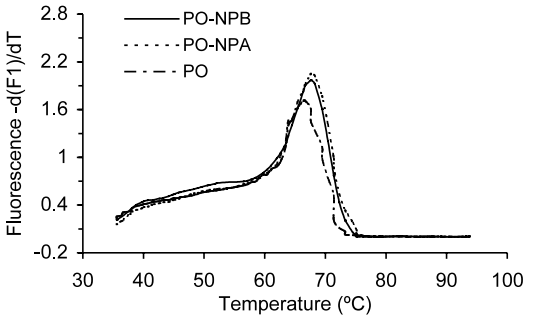

Fig. 8. Fluorescence melting curve analysis of control PO ($T_m = 66.33$ °C), PO-NPA ($T_m = 67.45$ °C), and PO-NPB ($T_m = 67.08$ °C).

4. Discussion

In the present study, we evaluated the ability of albumin nanoparticles to carry an antisense oligonucleotide adsorbed onto their surface and/or also incorporated into the matrix of the spheres. Thus, the forces driving the interaction albumin-PO were studied.

As expected from the presence of pH-sensitive end groups on albumin, the zeta potential of particle surface was strongly influenced by the pH of the dispersion medium (Table 1). Then, adsorption studies at different pH values were expected to provide further information about the attractive forces between oligonucleotides and nanoparticles (Balladur et al., 1997). At acidic pH, amino groups may induce a cationic charge on the surface whereas carboxylic groups would be in the uncharged form, hence phosphate groups on the PO interact well. Around pH 5, carboxylic groups are negatively charged (Elaissari et al., 1998) and their electronic state influenced the total surface charge that decreased the interaction between the oligonucleotide and the albumin. At basic pH conditions, the electrostatic interactions are much more disfavoured due not only to carboxyle groups which are negatively charged but also to amino groups which turned into the deprotonated form (Elaissari et al., 1998). Thus, the amount of adsorbed oligonucleotides was much higher at acidic pH. These results confirmed that pH sensitive groups located on the particle surface play a predominant role in oligonucleotide binding and correlate with the ionisation characteristics of the amino end groups. Moreover, the results pointed out that even at pH values at which the particles exhibit negative zeta potentials or the amino end groups are transformed to their deprotonated

Table 4
Evaluation of hybridization capability of the 21-mer oligonucleotide adsorbed or entrapped in albumin nanoparticles in the presence of snake venom phosphodiesterase (0.1 mg/ml) by duplex melting temperature determinations expressed as T_m values (°C)

Formulation	5 min (T_m values, °C)	60 min (T_m values, °C)	Overnight (T_m values, °C)
PO	NH[a]	NH	NH
PO-NPA	NH	NH	NH
PO-NPB	66.0 ± 0.1	63.9 ± 0.1	NH

PO, free oligonucleotide.
[a] NH, no hybridization capability.

form, there is a considerable amount of oligonucleotide still bound to the carrier. For certain pH values, one has to keep in mind that the zeta potential values do not directly reflect the amount of protonated amino end groups (Fritz et al., 1997). However, the adsorption of the PO at alkaline pH, when the amino groups are in their deprotonated form, can only be explained by the presence of additional hydrophobic interactions between the PO and albumin particles.

The desorption of the PO from the particle surface in media with different ionic strength or pH (Table 3) also confirmed that the binding of the PO to the particles was mediated by electrostatic interactions with the ionisable positive groups on the surface of the particles. With more alkaline pH ($7.4 > 6.5 > 5$), the proportion of charged amino groups decreased, the electrostatic forces involved in the binding disappeared and the desorption of higher amounts of PO was favoured. Concerning the effect of the ionic strength, high salt concentrations displaced PO adsorbed onto particles surface by screening the charged groups of both particle surface and PO phosphate groups, neutralising the electrostatic forces responsible for the interaction (Table 3) (Nakada et al., 1996).

It has to be noted that the adsorption of the oligonucleotide onto BSA nanoparticles was possible by the incubation of pre-formed particles with the oligonucleotide without the addition of any positive compound. This fact has to be seen as an advantage because any positive charge could cause toxicity and simplifies the formulation (Teixeira et al., 1999).

Usually, the adsorption of PO onto the surface of nanospheres has a dramatic effect on their zeta potential due to negative charges provided by the phosphate groups of the PO (Elaissari et al., 1994). In contrast, in the present case, the adsorption of increasing amounts of the PO slightly affect the zeta potential values of the particles. The effect was nil when the PO was incubated with the albumin prior to the formation of nanoparticles. For PO-NPB, a favourable hypothesis to explain this fact could be that an important part of the PO would be located inside the particles, rather than at their surface. A similar behaviour for PO-NPA, when the oligonucleotide remained adsorbed onto the particles, also suggests that the range of PO concentrations tested would be unable to modify the negative charge of the pre-formed nanoparticles. In fact, previous works reported that the adsorption of negative PO decreased the charge of positive particles (Fattal et al., 1998). However, in the present work unloaded nanoparticles were already negatively charged, unaffected by the ionisation state of the amino end groups which interact with the PO. So far, an important fraction of amino groups would still remain available to interact with more phosphate groups of PO molecules as also indicated by the following evidences: (i) the amount of PO associated to nanoparticles (adsorbed and/or incorporated) did not reach a maximum in the range of concentrations tested (Fig. 2); and (ii) the preparation of nanoparticles with increasing amounts of glutaraldehyde decreased the number of available amino groups and, however, it did not screen the association of the PO to the particles (Fig. 3).

The pH and ionic strength of the bulk solution controlled the desorption process of the PO from the particles because the interaction was mediated

by electrostatic forces (Table 3). A biphasic profile release when the PO was incubated with the albumin prior to the formation of the particles corroborated that, in this case, the PO was also incorporated into the matrix of the particles rather than adsorbed onto the particles, resulting in higher drug loadings (Fig. 2). Also this pattern of release indicated that the release of PO incorporated was directly dependent on particle degradation as confirmed by the fairly linear relationship (Fig. 7) between both phenomena. A similar pattern release from albumin particle systems have been described in earlier publications (Reddy et al., 1990). Then, the burst release corresponded to the desorption of the PO fraction onto the particles surface followed by a sustained release of that incorporated into the matrix, that resulted mainly from the erosion of the particles by the medium. Moreover, in this case, there is an incomplete release (about 20%) that could be atributted to hydrophobic irreversible interactions PO-particles not affected by a reduction of albumin charge. Such type of interaction did not happen when the oligonucleotide was incubated with the pre-formed nanoparticles.

Although it is difficult to anticipate the ideal release profile for a substance such as oligonucleotide, our results have shown that PO release is controlled by: (i) the effect of different media in the interaction BSA-PO; (ii) the preparation procedure (NPA or NPB); and (iii) mainly by the nanoparticle degradation.

The incorporation of PO into the matrix of albumin nanoparticles (PO-NPB systems) exposed the oligonucleotide to more aggressive conditions than its simple adsorption onto particles. However, none of both procedures induced any change in oligonucleotide hybridisation capability (Fig. 8) (Berton et al., 1999).

Moreover, the advantage of PO incorporated into albumin nanoparticles (PO-NPB) compared to other nanoparticulate strategies is that, as shown in the paper, the PO is entrapped into the matrix and not simply adsorbed onto the surface of the particles. As a consequence, PO-NPB exerted a better protection against degradation by nucleases (Table 4). In this way, it has been previously reported that, oligonucleotides adsorbed onto PIHCA (polyisohexylcyanoacrylate) nanoparticles through binding to CTAB (hydrophobic cation) were efficiently protected against enzymatic degradation even after 5 h incubation with phosphodiesterase (0.1 mg/ml) or in cell culture media (Chavany et al., 1992). Similar results were obtained by Zobel et al. (Zobel et al., 2000) with oligonucleotides adsorbed onto DEAE-dextran containing nanoparticles incubated with DNAase. Also Lambert et al. (Lambert et al., 2000) found that the encapsulation was much more efficient to protect oligonucleotides against degradation by serum nucleases that obtained by simple adsorption onto CTAB-coated nanospheres. However, the adsorption of the oligonucleotides in the systems previously reported was able to protect from enzymatic degradation in larger extension that obtained by the adsorption onto BSA nanoparticles. In our system, it could be caused, by the rapid desorption of the oligonucleotides from the nanoparticles surface, moreover promoted by a competition with buffer electrolites. When the oligonucleotide was encapsulated, the protection against degradation was well correlated with the degradation of BSA nanoparticles over time. A more rapid degradation of BSA nanoparticles (after overnight these carrier was completely degraded) as compared with other types of nanoparticles previously reported could explain our unfavourable results.

Thus, the results presented in this work show that albumin nanoparticles exert a certain protection against enzymatic degradation, one of the major obstacles for successfully antisense therapy. Also, albumin has been reported containing a fusogenic peptide able to disrupt endosomal membrane at low pH (Simoes et al., 2000). If the level of protection exerted by nanoparticles is able to improve its antiviral activity will be study in further experiments.

5. Conclusions

A great potential of albumin nanoparticles as carriers for oligonucleotides could be underlined from the following observations:

(i) The adsorption of the negatively charged oligonucleotides onto pre-formed BSA-

nanoparticles without addition of any positive compound is possible;

(ii) When the PO is incubated with the protein prior to the formation of particles, incorporation of the PO into the matrix of the carriers in addition to the adsorption onto their surfaces results in higher drug loading;

(iii) As indicated the effect of medium pH in the oligonucleotide adsorption (Table 1) and desorption (Table 3), the positive charges of amino groups located on the surface are predominantly responsible for the adsorption of negatively charged PO;

(iv) However, hydrophobic interactions not affected by the charge of particles play a role as deduced from the adsorption of the PO in media pH promoting negative nanoparticles (Table 1);

(v) BSA-nanoparticles could carry higher amounts of PO than described here because no plateau was reached; the drug loading was evaluated under unfavourable conditions as negatively charged free BSA or pre-formed nanoparticles, and in the presence of glutaraldehyde, a cross-linking agent that decreases the number of available amino groups;

(vi) The encapsulation of the PO inside the matrix of nanoparticles (PO-NPB) succesfully protected the oligonucleotide against enzymatic degradation by phosphodiesterase for 1 h at least.

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