Albumin Nanoparticles for the Intravitreal Delivery of Anticytomegaloviral Drugs

J.M. Irache*[1], M. Merodio[1], A. Arnedo[1], M.A. Camapanero[2], M. Mirshahi[3] and S. Espuelas[1]

[1]*Centro Galénico, Universidad de Navarra, Ap. 177, 31080-Pamplona, Spain*

[2]*Servicio de Farmacología Clínica, Clínica Universitaria de Navarra, 31080-Pamplona, Spain*

[3]*INSERM E.9912, Université Paris VI, 75006-Paris, France*

Abstract: Albumin nanoparticles (NP) were proved to be effective and safe carriers for delivering anticytomegaloviral compounds in the vitreous. NP improved the antiviral activity of both ganciclovir and the phosphodiester oligonucleotide analog to formivirsen. NP appeared to be fusogenic carriers able to target the nucleus of cells. In addition, these drug carriers were well tolerated when administered by the intravitreal route and did not induce autoimmune reactions.

Keywords: Albumin; nanoparticles, ganciclovir, fomivirsen; oligonucleotides, intravitreal delivery, retinitis.

1. INTRODUCTION

1.1. Human Cytomegalovirus (HCMV)

HCMV is a member of the Herpesviridae family of viruses, which are large DNA viruses that share the biological properties of latency and reactivation [1]. These viruses have a relatively restricted host range, long growth cycle and slow spread in cell culture. Approximately, 80% of the population in developing countries have detectable antibodies to HCMV [2]. However, the vast majority of HCMV primary infections in immunocompetent patients go clinically unrecognised, since they are associated with a mononucleosis-like syndrome. Only in patients with acquired immune deficiency syndrome (AIDS) and those immunocompromised due to hematopoietic stem cell transplantation or solid organ transplantation, HCMV is a major cause of morbidity and mortality [1,3,4].

The main clinical manifestations of the HCMV infection are gastrointestinal disorders, neurologic syndromes, pneumonia, hepatitis and retinitis [5]. HCMV retinitis is the most common opportunistic ocular infection and the major cause of visual loss in AIDS patients. Although with the advent of the highly active antiretroviral therapy, the incidence of HCMV retinitis has been reported significantly, 20 to 25% of patients with AIDS still develop cytomegalovirus retinitis during the course of their illness [6]. Without treatment this necrotizing retinitis progresses, resulting in irreversible blindness [7].

HCMV infection is also common in patients receiving solid organ transplants, and it is associated with increased morbidity as well as a risk of developing chronic rejection. Infection can be acquired post-operatively (from the transplanted organ) or from a re-activation of a latent disease [8]. HCMV has been linked to the development of atherosclerosis, arterial restenosis following angioplasty and solid organ transplant vascular sclerosis (TVS) [9,10]. HCMV infection doubles the 5- and 3-year rates of graft failure due to accelerated TVS in cardiac and liver transplant patients, respectively [11, 12]. In the absence of antiviral prophylaxis, symptomatic infections occur in approximately 39% of heart-lung recipients, 25% of heart transplants, 29% of liver transplants, and in 8% of patients receiving renal transplants [13-16]. Without prophilaxis, infections primarily occur in the first three months post-transplantation; retinitis, on the other hand, typically appears more than 6 months after the transplantation [17].

In retinitis, the endothelial cells of the ocular vessels, as well as monocytes and granulocytes, are the major target sites for the viral latency and haematological spread whereas the fibroblasts play a minor role in the multiplication and spread of the virus in infected tissues [18]. Currently, there are four FDA-approved drugs for the treatment of HCMV retinitis: ganciclovir, foscarnet, cidofovir and fomivirsen.

1.2. Ganciclovir (GCV)

Ganciclovir (9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine) is one of the most used drugs in the treatment of HCMV infections [4,19]. This drug prevents the replication of the viral DNA but does not eliminate the virus from the tissue; so long term therapy is necessary to control the disease [20]. In the cell, this antiviral compound is firstly phosphorylated by a viral enzyme [21], and transformed into ganciclovir triphosphate by other two cellular guanylate kinases. This ganciclovir derivative interacts with the viral DNA synthesis, resulting in the suppression of the DNA chain elongation at ganciclovir concentrations normally lower than 10 μM [22,23].

The administration of intravenous ganciclovir is effective at slowing the progression of HCMV retinitis; although long-term treatment (usually for 14 to 21 days) and high-dose intravenous injections have caused most side-effects including severe neutropenia and trombocytopenia [24-26]. These side-effects are dose-related and are usually reversible on withdrawal of the drug. Moreover, in patients receiving chronic maintenance therapy, HCMV resistance to the treatment can emerge [27, 28]. For these reasons, local

*Address correspondence to this author at the Centro Galénico, Department of Pharmaceutical Tecnology, University of Navarra, Ap. 177, Irunlarrea, 1, 31008 Pamplona, Spain. Tel: ++34 948 425600; Fax: ++34 948 425649; E-mail: jmirache@unav.es

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intravitreal therapy has been proposed as a safe and well-tolerated alternative route for the treatment of severe retinitis infection. Intravitreal administration produces high ganciclovir concentrations in the intravitreal fluid, apparently with minimal systemic absorption. The half-life of ganciclovir in vitreous fluid following intravitreal administration is about 13 hours [29] and, thus, frequent injections are necessary to maintain the therapeutic levels. This fact limits its use because these injections are associated with a risk of cataract development, retinal detachment and endophtalmitis [30,31].

In order to overcome these drawbacks and to improve patient comfort by reducing the frequency of dosing, many drug delivery systems able to provide therapeutic ganciclovir concentrations for prolonged periods of time have been proposed, including implantable devices [32,33], liposomes [34] and microparticles [35]. Ganciclovir intravitreal implants continuously release drug for 6 to 8 months into the vitreous at levels substantially higher than those achieved with intravenous therapy [36,37]. However, this device is not biodegradable and requires a surgical procedure for its removal or repeated implantation [38]. Furthermore, those systems may produce long-term side-effects, such as astigmatism or vitreous haemorrhages [32].

Another way to minimise these problems could consist of administering ganciclovir in biodegradable carriers (i.e. liposomes and microparticles). These new pharmaceutical dosage forms appear to preserve the visual acuity and quality of life of patients; although it has been described that an intravitreal injection of particulate or vesicle dispersions requires submicronic size ranges to avoid interference with vision [39]. On the one hand, liposomal formulations can provide effective intravitreal concentrations of ganciclovir for 30 days with no sign of ocular toxicity [34]. On the other hand, microparticles containing ganciclovir did not show any retinal or adjacent tissue toxicity following either electroretinography or histological studies [35]**.** In addition, this formulation was able to release a daily quantity of ganciclovir within the therapeutic range for at least 14 days [40].

1.3. Fomivirsen (PS)

Fomivirsen sodium is a 21-base phosphorothioate oligodeoxynucleotide complementary to the mRNA of the major immediate-early region proteins of human cytomegalovirus, and is a potent and selective antiviral agent for cytomegalovirus retinitis via an antisense mechanism [41,42]. It was the first drug of the new antisense oligonucleotides to be evaluated and approved by the FDA for the treatment of AIDS-related cytomegalovirus retinitis [43]. In the vitreous, fomivirsen is slowly cleared with a half-life of about 55 hours [44]. Preclinical studies show that, after intravitreal administration, this drug distributes to retina and is slowly metabolised by exonuclease digestion [44,45].

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 [46]. However, crucial problems such as the stability of oligonucleotides in relation with nuclease activity and the low intracellular penetration have to be solved. 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 sulphur atom [46]. These phosphorothioates, including fomivirsen, and many other oligomer classes that are experimentally employed, possess a high stability against degradation induced by nucleases. However, their capacity to penetrate across membranes remains poor due to their polyanionic structure [47]. In addition, chemical modifications of oligonucleotides induce both a loss of the antisense activity [48] and an increase in their ability to interact in a non-sequence-specific manner with other macromolecules, including key structural proteins, enzymes, receptors or growth factors [49-51]. At high concentrations they can even inhibit RNase H [52]. Due to these non-antisense effects, unmodified phosphodiester oligonucleotides would be more interesting for developing safe treatments.

Another possibility may consist in the use of colloidal carriers to both protect unmodified oligonucleotides against enzymatic degradation and, in some cases, promote their cellular uptake. Lipid vesicles have been the most widely exploited, including cationic liposomes [53], virosomes [54], pH sensitive- [55], fusogenic- [56] and immuno-liposomes [57]. In spite of some advantages offered by these carriers, their formulation and preparation are complicated and, moreover, they display a relatively low stability in the presence of serum [50]. Similarly, biodegradable nanoparticles have also been studied as potential inert and biocompatible carriers for genetic materials. In most cases, the coating of these particles with cationic polymers was required to obtain successful interactions with the negatively charged oligonucleotide molecules. In this way, electrostatic adsorption of oligonucleotides on poly(alkylcyanoacrylate) nanoparticles was possible by their coating with positively charged chemicals such as cetyltrimethylammonium bromide [58], dextran derivatives [59] or cationic lipids [60]. More recently, formulations able to encapsulate oligonucleotides rather than a simple electrostatic adsorption have also been developed, such as poly(isobutylcyanoacrylate) nanocapsules [48] and poly(lactic acid) nanoparticles [47]. As compared with the simple adsorption, the oligonucleotide encapsulation improves its protection against enzymatic degradation. However, these encapsulating-carriers are difficult to obtain and require the use of aggressive conditions that compromise the oligonucleotide stability.

The present work reviews the properties of albumin nanoparticles as carriers for the intravitreal delivery of two different anticytomegaloviral drugs: ganciclovir and a 21-mer phosphodiester oligonucleotide analogue to fomivirsen (PO). In addition, this work includes studies of intraocular disposition and toxicity after intravitreal injection in rats.

Nanoparticles are submicronic (< 1 μm) systems generally made of synthetic or natural polymers (biodegradable or not). According to the process used for the preparation of the nanoparticles, nanospheres or nanocapsules can be obtained. Unlike nanocapsules (vesicular systems in

which the drug is confined to an aqueous or oily cavity surrounded by a single polymeric membrane), nanospheres are matrix systems in which the drug is dispersed throughout the particles or/and adsorbed to their surface [61]. In recent years, biodegradable polymeric nanoparticles have attracted considerable attention as potential drug delivery devices in view of their applications in the controlled release of drugs, including the new classes of active molecules such as peptides, proteins, genes and oligonucleotides [62,63]. If designed appropriately, nanoparticles may be able to modify the distribution of an associated substance. They can therefore be used to improve the therapeutic index of drugs by increasing their efficacy and/or reducing their toxicity [61,64].

Albumin nanoparticles may be interesting delivery systems for the intravitreal administration of drugs. They show a smaller size compared to microparticles and, in general, better controlled release properties than liposomes which may contribute to the preparation of a more efficacious and secure pharmaceutical dosage form and, thus, improve patient acceptance and compliance. These carriers have been extensively studied in previous works as suitable for drug delivery [65-67] since they are biodegradable [68], non-toxic [69] and non antigenic [70]. Because of their defined primary structure and high content of charged amino acids, the albumin-based nanoparticles could allow the electrostatic adsorption of positively (i.e. GCV) or negatively charged (i.e., oligonucleotide [71]) molecules without the requirement of other compounds. In addition, protein nanoparticles can be easily prepared under soft conditions, by coacervation or controlled desolvation processes [65].

2. ALBUMIN NANOPARTICLES AS CARRIERS FOR GANCICLOVIR

Albumin nanoparticles containing ganciclovir were prepared by a coacervation method and chemical cross-linkage with glutaraldehyde. For this purpose, an aqueous solution of the protein was desolvated with ethanol to induce the formation of small aggregates of albumin (coacervates), which were subsequently treated with glutaraldehyde. These nanoparticles were purified by centrifugation and freeze-dried using manitol as cryoprotector. Depending on the step in which the ganciclovir was added, two different formulations (called GCV-NPA and GCV-NPB) were evaluated. For GCV-NPA, the antiviral was directly bound to the surface of empty nanoparticles. For GCV-NPB, ganciclovir was incubated with the protein prior to the formation of nanoparticles by coacervation. "(Fig. **1**)" shows a schematic representation of the two preparative processes.

The size of the different ganciclovir-loaded nanoparticle formulations was comprised between 200 and 300 nm and the antiviral loading was calculated to be around 27 μg GCV/mg nanoparticles for GCV-NPB, and 15 μg/mg for GCV-NPA. The *in vitro* release profiles of ganciclovir from the nanoparticles showed a biphasic pattern, characterised by an initial and rapid release period (burst effect) followed by a step of slower release for up to 10 days "(Fig **2**)". For GCV-NPA, about 60% of the loaded drug was released in the first 24-h, whereas for GCV-NPB, this burst effect was calculated to be only around 40%. After 10 days, about 90% and 60% of the loaded antiviral was released from GCV-NPA and GCV-NPB, respectively.

These release profiles were related to the presence of two different ganciclovir fractions in the nanoparticles. The former could show weak interactions with the nanoparticulate dosage form and may be responsible for the initial ganciclovir release during the first day. Therefore, the higher drug release from GCV-NPA may be due to the rapid desorption of the adsorbed drug fraction. The latter release could be due to the ganciclovir fraction covalently linked to the protein matrix via glutaraldehyde [67]. This covalent linkage between the antiviral and the protein was confirmed by thin layer chromatography experiments and release studies under acidic and basic conditions [67].

The ability of albumin nanoparticles to modify the antiviral activity of ganciclovir was evaluated in human embryonic lung fibroblasts (MRC-5) and human corneal fibroblasts (CHN). MRC-5 cell line has been widely employed for antiviral screening tests [23,72,73] whereas corneal stromal cells, as well as primary cornea-derived cell lines, have demonstrated permissiveness to HCMV replication [74]. These cells were infected with the HCMV strain Ad169 and, after infection, incubated with different concentrations of ganciclovir either dissolved in water

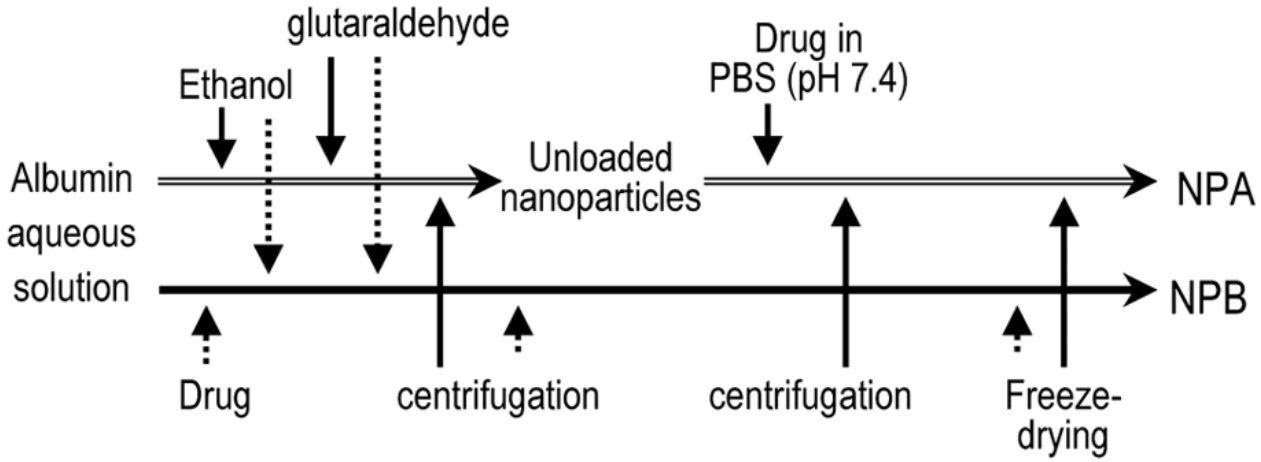

Fig. (1). Schematic representation of the different ganciclovir-loaded albumin nanoparticle formulations.

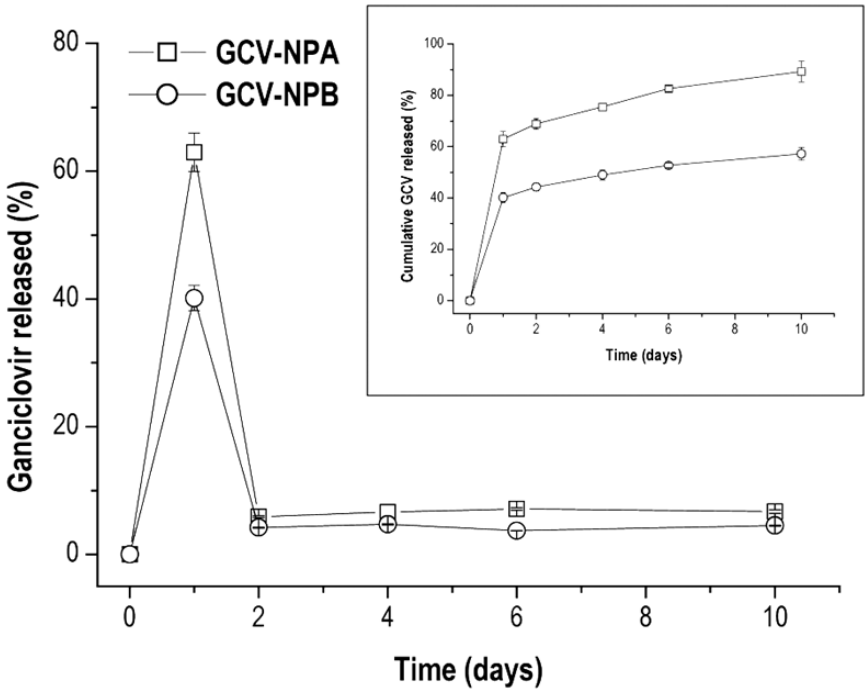

Fig. (2). *In vitro* release of ganciclovir from the different nanoparticulate formulations in complete RPMI-culture medium at 37ºC.

(GCV) or loaded in albumin nanoparticles (GCV-NPA or GCV-NPB). Two different tests were used for the evaluation of the anti-HCMV activity: plaque reduction assay (PR) on day 9 post-infection, and quantification of early antigen expression (AE) on day 5-postinfection. PR reveals the efficacy of the treatment at the end of the viral replication cycle [75,76]. On the contrary, AE permits the quantification of the secretion of antigens before DNA synthesis [73].

"(Fig. **3**)" shows the antiviral activity (expressed as IC_{50}) of ganciclovir formulations in the HCMV replication on both MRC-5 and CHN cultures when cells were infected with 100 pfu per well. By plaque reduction assay, the effectiveness of all formulations appeared to be lower (around 2-4 times) in CHN cultures than in MRC-5 infected cells. Whatever cell line or assay tested, GCV-NPA was the most active formulation whereas GCV-NPB showed an anti-

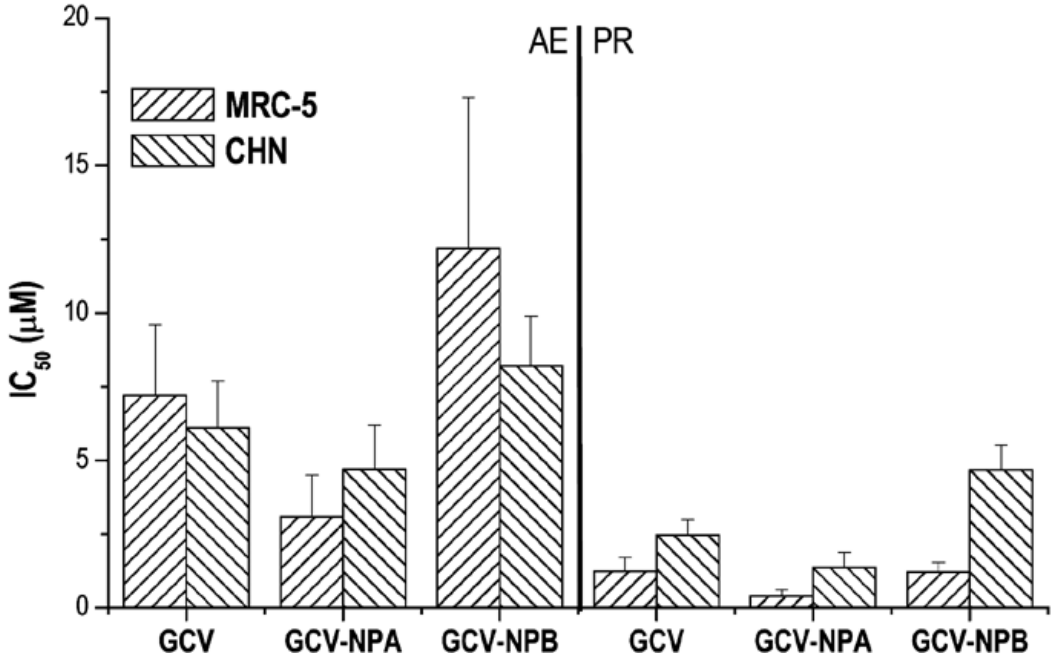

Fig. (3). Activities of the different ganciclovir formulations on MRC-5 and CHN. The activity was measured by both plaque formation assay (PR) and early antigen expression (AE) and expressed as the concentration needed to either reduce plaque formation or antigen expression by 50% when compared with a non-treated control (IC_{50}). Infection was performed with 100 pfu HCMV strain Ad169 per well.

HCMV effect similar or lower to that observed for the drug aqueous solution. In fact, GCV-NPA inhibited the viral replication with a 3-fold efficacy and nearly 2-fold higher than the drug aqueous solution for MRC-5 and CHN, respectively. Other works also revealed a substantially greater activity (3.5-fold) for the loading of ganciclovir into colloidal carriers such as liposomes [77]. Similarly, the estimated IC_{50} value of the ganciclovir aqueous solution in MRC-5 cells was of the same order as previously reported by other authors [23,78].

The GCV-NPA formulation also inhibited the expression of the HCMV early antigens at a lower concentration than the conventional drug solution, increasing the efficacy by 57% and 22% in MRC-5 and CHN cells, respectively. For MRC-5 cells, the IC_{50} for GCV-NPA was between 2 and 4 times lower than for GCV and GCV-NPB, respectively "(Fig. **3**)". For CHN cells, the estimated IC_{50} value of GCV-NPA was 1.5- and 2-fold lower than GCV and GCV-NPB, respectively "(Fig **3**)". However, the ganciclovir levels required to obtain the same antiviral efficiency was up to 10 times higher than that estimated by PR. This result can be attributed to methodological changes. In fact, AE allows us to estimate the expression of viral proteins when the amount of viral DNA synthesised is very low. Because of this, the ganciclovir effect during the first events in the replication cycle of HCMV is limited [79]. In addition, this small quantity of viral DNA synthesised in the presence of ganciclovir may be able to function as a template for the transcription of the subsequent antigens implied in the HCMV replication cycle and novel early antigens [79]. As a result, the inhibition of the antigen expression requires significantly greater drug concentrations than those for the inhibition of HCMV replication [73,80].

Interestingly, the antiviral effect of all ganciclovir formulations was more relevant when they were added with a delay of 48 h than immediately post-infection, as previously reported with the free drug [76]. The IC_{50} was reduced 3.5-times for GCV-NPA, 2.6- for GCV-NPB and twice for the drug aqueous solution [81]. The slow replication cycle of the HCMV can be one of the possible explanations. The complete expression of viral antigens and the yield of detectable levels of progeny virus require 48 to 96 h [79]. These processes determine (i) the induction of the enzyme responsible for the initial phosphorilation of ganciclovir into the active metabolite, which requires DNA replication for maximum expression, and (ii) the synthesis of the DNA polymerase, inhibited by the ganciclovir triphosphate. Thus, the addition of ganciclovir 48 h post-infection lead to a higher drug retention by its metabolisation and, therefore, an enhancement in the inhibition of the viral DNA polymerase [81]. Moreover, all the ganciclovir formulations displayed highly significant lower IC_{50} values when the cells were infected with a less concentrated viral inoculum (10 pfu/well *vs.* 100 pfu/well) ($p < 0.001$) [81].

In any case, the results of antiviral activities seemed to be directly correlated to the drug internalisation by infected

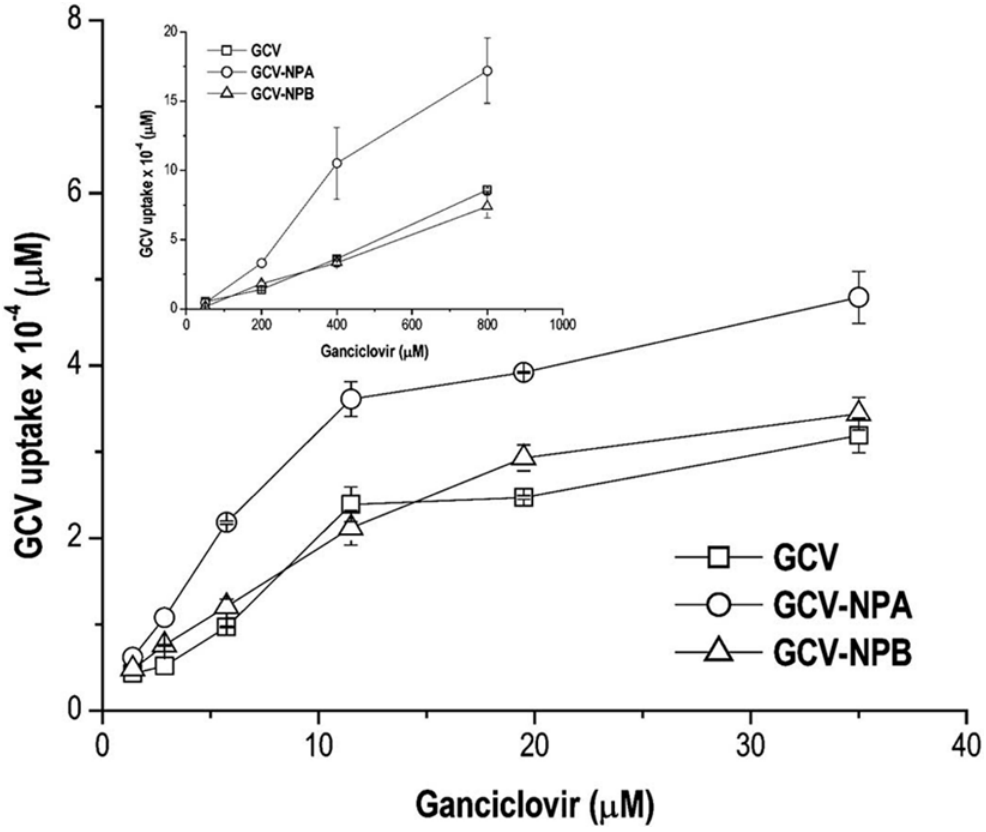

Fig. (4). Ganciclovir uptake by HCMV infected-CHN cells as a function of drug concentration. The inset is a plot of the ganciclovir uptake by non-infected CHN cells. Experimental conditions: $2x10^4$ cells/cm^2 were incubated for 96 h at 37°C. Infection was performed with 10 pfu HCMV strain Ad169 per well.

cells [81,82]. Thus, GCV-NPA (the most active formulation) highly improved the drug uptake by CHN cells, whereas GCV-NPB led to a drug internalisation similar to the ganciclovir aqueous solution "(Fig. **4**)". In addition, the amount of ganciclovir accumulated in the cells would be also related with its release profile from the nanoparticles (see "Fig. **2**)".

In principle, the entry of ganciclovir into the cells may be explained by two simultaneous mechanisms: passive diffusion of the free drug and endocitosis of the nanoparticles containing the antiviral. On the one hand, nanoparticles can be taken up by a number of different cells [83,84]. On the other hand, ganciclovir may entry in the cells by a passive diffusion mechanism [85]. As GCV-NPA can release the antiviral in a more rapid way than GCV-NPB, this formulation would be more efficient to accumulate ganciclovir inside the cells than either GCV-NP or an aqueous solution of the drug. On the other hand, for all the formulations, the ganciclovir uptake was more efficient in infected than in non-infected fibroblasts "(Fig. **4**)". The ganciclovir mechanism of action led to a higher accumulation of the active compound, ganciclovir-5'-triphosphate, in infected cells so the entry of the drug was enhanced to follow the subsequent phosphorilative reactions. When the formation of the triphosphate metabolic compound was low, as it occurred in non-infected cells, the internalised drug would be a poor substrate to be retained inside the cell, and tended to move out [86].

"(Fig. **5**)" shows the antiviral activity of ganciclovir formulations in the replication of a clinical HCMV isolate on MRC-5 cultures when cells were infected with 20 pfu per well. As estimated from the sigmoidal fit of experimental data, it was clear that again GCV-NPA was found to be the most active formulation (IC_{50} of about 0.3 μM) whereas the activity of the antiviral when loaded in GCV-NPB was found to be 2.5-times lower than for the antiviral aqueous solution (GCV).

The improvement of the antiviral activity of ganciclovir when loaded in nanoparticle formulations was not related to any cytotoxic effect. The drug concentration interfering in the normal cell proliferation easily surpassed the antiviral concentrations, as has been observed by other authors [80]. At these levels (higher than 100-200 μM), the amount of ganciclovir-triphosphate is enough to inhibit the cellular DNA polymerase. Both Trypan Blue staining and Hoechst 33342 DNA-binding revealed that the drug adsorption in albumin nanoparticles (GCV-NPA), which provided the greater cellular internalisation of the antiviral, led to a severe increase of the drug antiproliferative effect when compared to the free drug. On the contrary, its loading in albumin nanoparticles (GCV-NPB) clearly reduced this adverse effect [81]. Similarly, at a drug concentration higher than 200 μM, the ganciclovir formulations also induced a certain damage to the cell membrane, as revealed with the lactate dehydrogenase assay. Again, the higher toxicity was obtained by GCV-NPA whereas GCV-NPB ended up as the least toxic formulation. On the other hand, unloaded albumin nanoparticles did not induce a significant inhibition on fibroblast proliferation with concentrations as high as 20 mg/ml, confirming the harmlessness of these carriers for the treatment of HCMV infections.

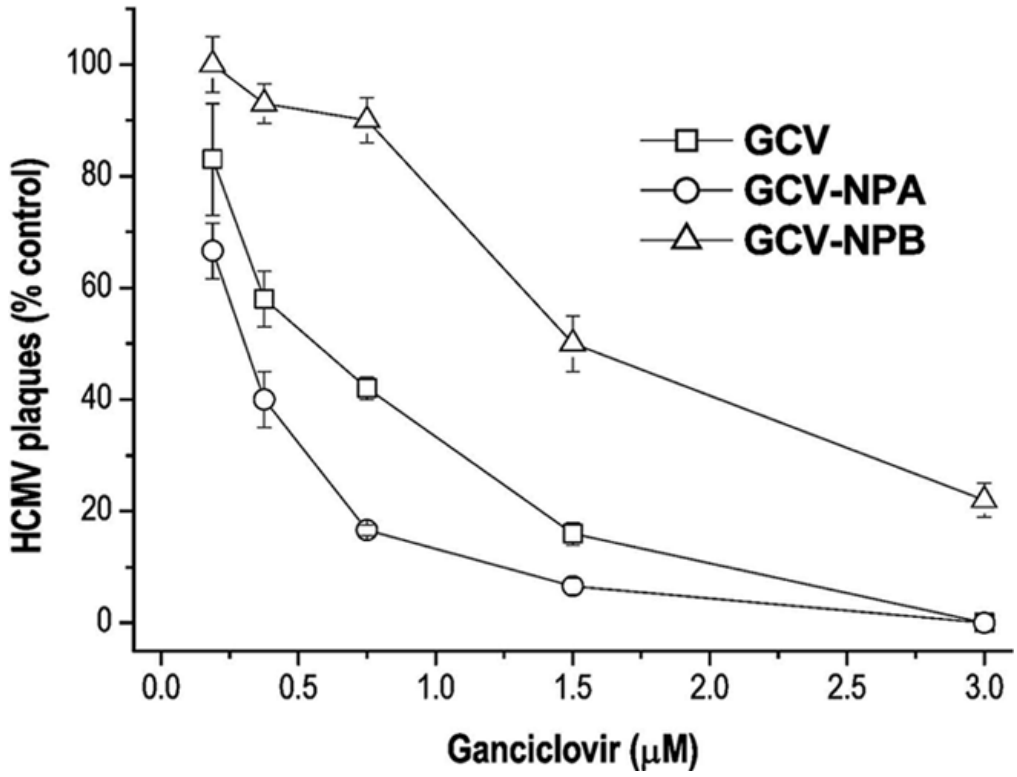

Fig. (5). Effect of different formulations of ganciclovir in the replication of a clinical isolate of HCMV on CHN cells. Experimental conditions: the cells were infected with 20 pfu/well and the treatments were added just after infection.

Table 1. Physicochemical Characteristics of Albumin Carriers. The Different Formulations were Prepared with an Oligonucleotide/Albumin Ratio of 5 μg/mg. Data Express the Mean±sd

Formulation	Size (nm)	Zeta Potential (mV)	Drug loading (μg/mg NP)	Entrapment efficacy (%)
Empty 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

3. ALBUMIN NANOPARTICLES AS CARRIERS FOR AN ANTIVIRAL ANTISENSE OLIGONUCLEOTIDE

Phosphodiester (PO; a 21-mer analogue to PS) and phosphorotioate (PS; fomivirsen) oligonucleotides were either adsorbed or incorporated in albumin nanoparticles following the two procedures described before (see "(Fig. **1**)". In the former, oligonucleotide-loaded nanoparticles were obtained by the incubation of the antisense oligonucleotide with empty nanoparticles for 2 h at room temperature (pH 7.4, 0.05 mM) (PO-NPA or PS-NPA). In the latter, the oligonucleotide was incubated with the albumin aqueous solution (2% w/w) for 2 h at room temperature prior to the formation of the nanoparticles by coacervation (PO-NPB or PS-NPB).

The main physico-chemical characteristics of the different formulations are summarised in (Table **1**). In all cases, all batches displayed a similar size (around 250 nm) and negative surface charge (about -22 mV). However, when the oligonucleotide was incubated with the protein before nanoparticle formation (PO-NPB, PS-NPB) the drug loading was higher than when adsorbed on the surface of empty nanoparticles (PO-NPA, PS-NPA). On the other hand, PS displayed a higher affinity for albumin nanoparticles than PO. Thus, NPA were able to load 4.5-times more PS than PO (3.4 μg/mg vs. 0.75 μg/mg). Similarly, for oligonucleotide entrapped in nanoparticles (NPB), the loading of PS and PO were 5.24 and 4.67 μg oligonucleotide/mg nanoparticle, respectively (Table **1**). All of these results can be explained by the capacity of phosphorotioate oligonucleotides to interact much more strongly with proteins than phosphodiester oligonucleotides [87]. This is probably due to the lower electronegativity of the sulphur atom (located in the phosphodiester backbone) in comparison with the oxygen one.

Previous studies revealed that these nanoparticle carriers showed a similar release profile characterised by an initial step of rapid release of an important fraction of the loaded drug (burst effect), followed by a step in which the remaining oligonucleotide was released in a sustained way [88]. In any case, NPB displayed a higher ability than NPA to prolong the release of the oligonucleotide over a period of time. Thus, NPA formulations were exhausted after 8 hours whereas NPB formulations were able to maintain the release of the loaded oligonucleotide for up 5 days (not shown).

On the other hand, both PO-NPA and PO-NPB formulations displayed release profiles greatly dependent on the pH and ionic strength of the release medium. As shown in (Table **2**), the amount of PO released from albumin nanoparticles increased by raising the pH of the release medium over the range between pH 5 and 7.4. In acidic conditions the burst release effect 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. Similarly, by increasing the ionic strength of the release medium, the burst effect dramatically increased: the initial release raised from 35% of the loaded oligonucleotide when disposed in PBS 0.05M, to 60% when the release medium was PBS 1M. For NPB nanoparticles, a direct relationship between the cumulative amount of the oligonucleotide released vs. time and the degradation of nanoparticles in the course of time was found [88]. This fact indicated that the effect of different media in albumin degradation was also an important factor controlling the release of the oligonucleotide incorporated into the nanoparticles. In that sense, the preparation of albumin nanoparticles with a higher amount of glutaraldehyde slightly decreased the rate of carrier degradation and, thus, the oligonucleotide release.

Table 2. Influence of the pH and Ionic Strength on the Immediate Release of PO From PO-NPA and PO-NPB Formulations

Buffer solution		PO-NPA (% PO released)	PO-NPB (% PO released)
pH	Ionic strength		
5	0.15 M	11.96±0.25	15.87±0.71
6.5	0.15 M	31.66±2.02	31.83±0.20
7.4	0.05 M	13.80±0.15	34.15±1.07
	0.15 M	48.07±2.72	46.17±0.35
	1.00 M	90.69±1.54	59.87±0.24

The processes of nanoparticle preparation did not affect the hybridisation capability of the oligonucleotide when loaded in albumin carriers [88]. Similarly, no difference was found between free PS or PS loaded into albumin particles. However, free PO and PO-NPA lost its hybridisation capability immediately after a 5 min incubation period with phosphodiesterase at 0.1 mg/mL. Only the encapsulation of the oligonucleotide inside the matrix of the nanoparticle was able to maintain the oligonucleotide hybridisation capability

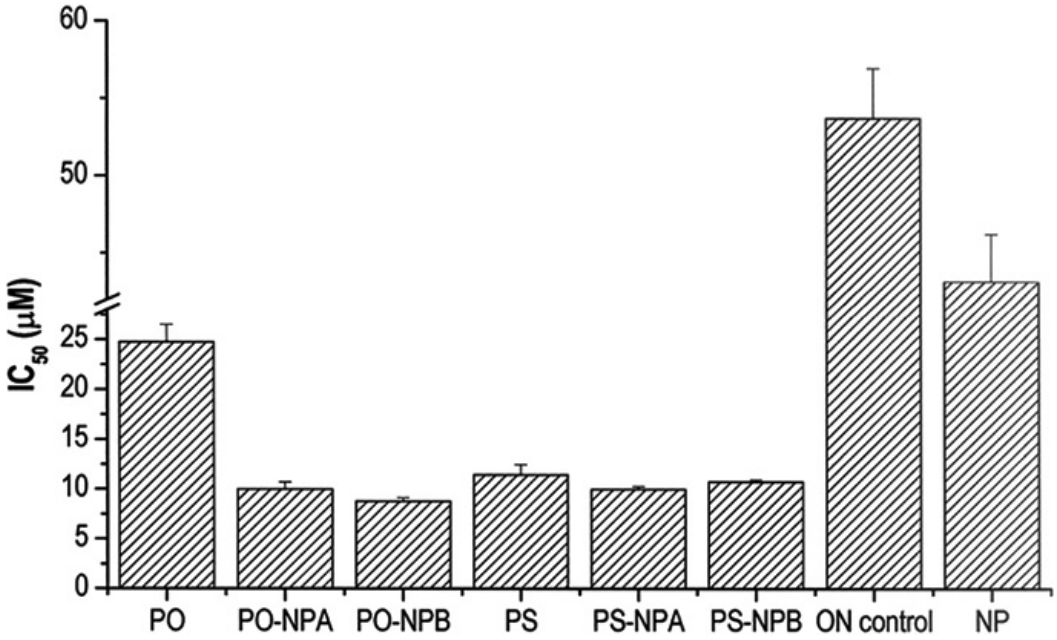

Fig. (6). Effect of the different formulations of oligonucleotides in the HCMV replication on MRC-5 cells by plaque reduction assay. Data express the concentration needed to reduce plaque formation by 50% (IC_{50}). Activities of unloaded nanoparticles (NP) and a control oligonucleotide (ON control) are shown for comparison. Cells were infected with 200 pfu HCMV RC256 strain per well.

after incubation with the enzyme. Lambert *et al*. [89] also found that the encapsulation was much more efficient at protecting oligonucleotides against degradation by serum nucleases than the simple adsorption to the surface of nanoparticles. This fact may be explained by the rapid desorption of the oligonucleotide from the surface of nanoparticles, promoted by a competition with electrolytes. When the oligonucleotide was encapsulated, the protection against degradation was well correlated with the degradation of albumin nanoparticles over time [88].

The antiviral activity of the different formulations was tested by plaque reduction assay. For this purpose, MRC-5 cells were infected with 200 pfu HCMV RC256 strain per well. After infection, the cells were incubated with the oligonucleotide formulations for 3 days. On day 9 post-infection, the antiviral activity was calculated as the ratio between the number of plaques in the treated wells and in the control wells. "(Fig. **6**)" shows the IC_{50} value of the different formulations tested. Under these experimental conditions, PO showed a low antiviral activity; although its adsorption or encapsulation in NP significantly increased its antiviral activity ($p<0.001$). In fact, the PO loading in NP inhibited the HCMV replication with an efficacy between 2.4- and 3-fold higher than the free PO. In addition, PO-NPB showed a slightly higher efficiency than PO-NPA when comparing IC_{50} data (8.80 μM vs. 10.0 μM). On the other hand, PS exhibited significantly higher activity than PO ($p<0.001$); although its association/encapsulation in NP only induced a very low improvement of its antiviral activity (see "Fig. **6**)". Interestingly, the use of albumin nanoparticles to load PO allowed us to obtain an anti-cytomegaloviral activity similar to the PS derivative ($p<0.05$). In addition, this antiviral activity was not only due to oligonucleotide induced cytotoxicity since effects on cell viability were only observed at concentrations higher than 20 μM (not shown).

This improvement in the antiviral efficacy of PO appeared to be related to the capacity of albumin nanoparticles to promote its entry into the nucleus of the cells "(Fig. **7**)". When MRC-5 cells were incubated for 24 h at 37ºC with free FITC-PO, a perinuclear punctuate pattern corresponding to oligonucleotide accumulation within vesicular compartments such as endosomes and/or lysosomes was observed. In addition, no detectable nuclear accumulation was found "(Fig. **7a**)". On the contrary, PO loaded in albumin nanoparticles showed a homogeneous fluorescence located in the cell nucleus "(Fig. **7b**)".

These results can be explained by the possibility that these carriers would display fusogenic properties, which permitted the targeting of the cell nucleus by the oligonucleotide. These results agree well with previous findings concerning higher transfection efficiencies of lipoplexes when coated with albumin [90]. In fact, albumin possesses a fusion peptide, termed P-9 (Phe-Ala-Glu-Asp-Lys), with a molecular weight of about 9200, composed of three amphiphilic helixes, which form a hydrophobic domain [91,92]. Conformational changes induced by acidification implies a partial opening of the albumin molecule facilitating the exposition of the P-9 hydrophobic domains to membranes, key features for interaction of the peptides with the endosomal membrane and for inducing membrane fusion [93,94].

Figure. **8** shows a schematic representation of the major mechanisms involved in the internalisation of an oligonucleotide when dissolved in an aqueous solution (free PO) or loaded in albumin nanoparticles. The antisense oligonucleotide, in order to perform its inhibitory task, has

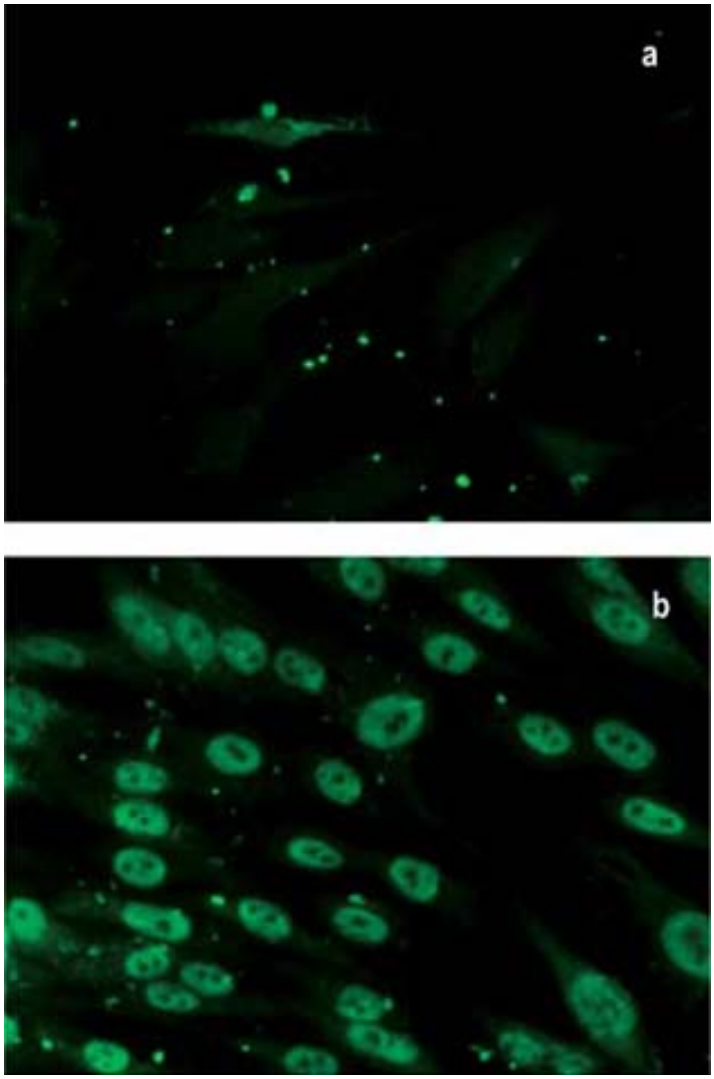

Fig. (7). Influence of albumin nanoparticles on the subcellular distribution pattern of FITC-PO in MRC-5 cells by fluorescence microscopy. MRC-5 cells were incubated with 5 μM oligonucleotide for 24 h at 37ºC. (a) FITC-labelled PO; (b) FITC-labelled PO-NPB.

to pass through the cell membrane, escape from the cytoplasm vesicles and, then, reach the target in either the cytoplasm or the nucleus [95]. The cell membrane is a potent natural barrier to large and negatively charged molecules, as are the oligonucleotide [96] (route 1 in "(Fig. **8a**)". However, it appears that oligonucleotides may enter in the cells by endocytosis (routes 2 and 3 in "(Fig. **8a**)" [97,98] via a number of cell surface "receptor-like" proteins [99]. Once in the cell, oligonucleotides accumulate in endosomes and lysosomes [100] where they would be mainly degraded by nucleases. On the other hand, albumin nanoparticles containing the antisense oligonucleotide would also enter in the cell by endocytosis [101,102]. Then these carriers would be located in endosomes (see "Fig. **8b**)" where albumin nanoparticles would protect PO from its enzymatic degradation. However, the modification of the environmental conditions within the endosomal compartment would induce conformational changes of albumin molecules forming the nanoparticles and their interaction with the endosomal membrane. This fact would enhance the permeability and/or destabilisation of this membrane, and, as a result, promote the cytosolic delivery of the antisense oligonucleotide.

4. OCULAR DISPOSITION AND TOLERANCE OF ALBUMIN NANOPARTICLES AFTER INTRAVITREAL INJECTION

The distribution of albumin nanoparticles in the eye was witnessed in animals injected with nanoparticles labelled with biotin (BIO-NP). For this purpose, the albumin solution used to prepare the empty albumin nanoparticles (see "Fig. **1**)" contained a mixture of native albumin and biotin-labelled protein in a ratio of 15:1 w/w. Then the nanoparticles were prepared as described before. The resulting carriers displayed a slightly higher size than conventional nanoparticles (304 vs. 250 nm); although, their surface properties (i.e. negative charge) were similar.

BIO-NP were dispersed in saline and administered by the intravitreal route to laboratory animals (900 μg dispersed in 5 μL). Two weeks after this single injection to rats, the carriers appeared to diffuse through the vitreous space. In

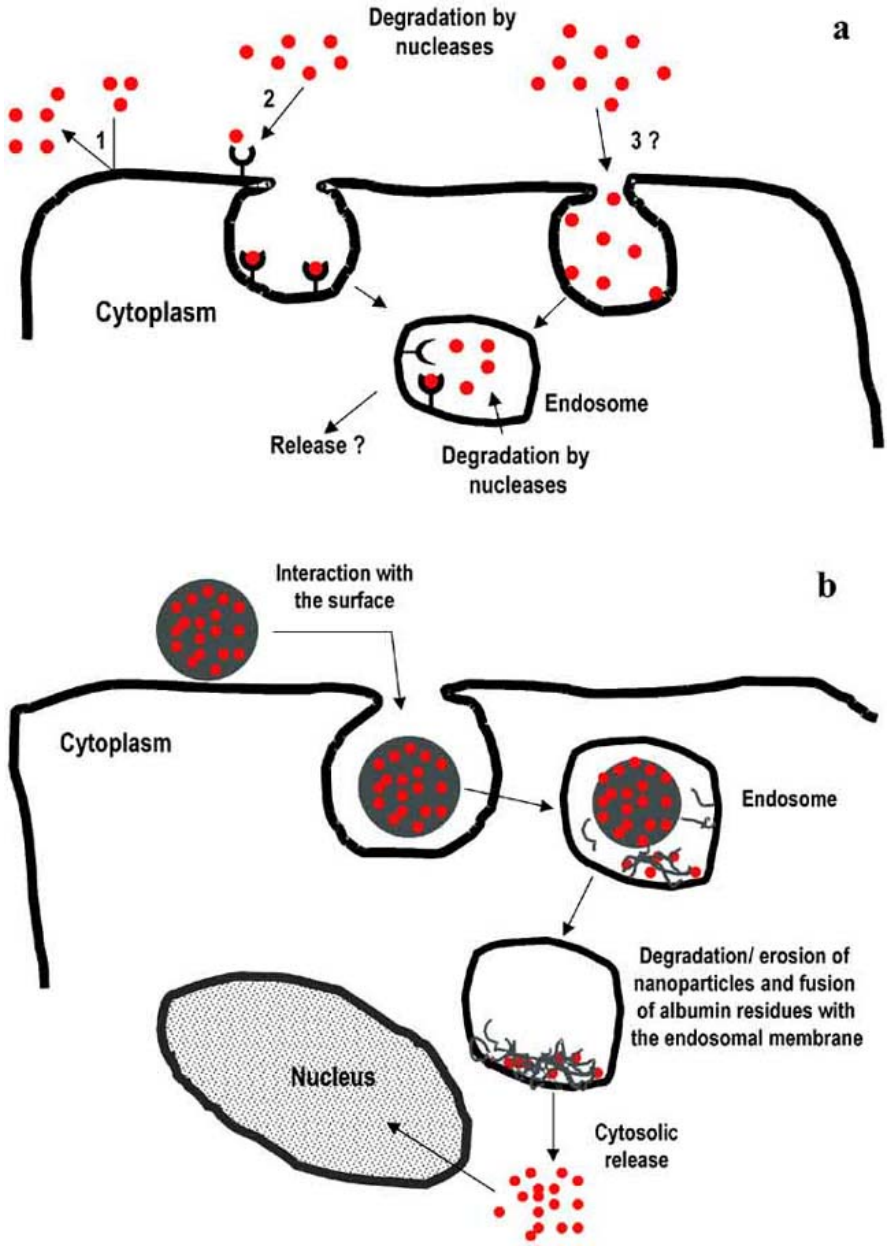

Fig. (8). Schematic representation of the major mechanisms involved in the internalisation of an oligonucleotide when dissolved in an aqueous solution (free PO) or loaded in albumin nanoparticles.

fact, a significant amount was mainly found in a thin layer overlying the retina "(Fig. **9a**)". Furthermore, nanoparticles were also found in adjacent sites to the blood-aqueous barrier and in the ciliary body "(Fig. **9b**)".

Similarly, minor foci of nanoparticles were observed between the stroma and the corneal epithelium (not shown). These results can be related to the high porosity of these ocular organs and, probably also, by an elimination mechanism of the particles via the ophthalmic anterior chamber. This route of elimination has been described for macromolecules and takes several days to be completed [101]. Finally, it is interesting to note that no evidence of nanoparticles was observed in the inner retinal layers (i.e. visual cells and neuronal interplay area). These results are in accordance with previous results concerning poly(lactic-co-glicolic) microparticles that also demonstrated a certain ability to reach the retinal layers [102,103].

Figure **10** shows the histological evaluation of the different ocular tissues after the intravitreal administration of NP. Two weeks post-administration, the histopathological analysis confirmed the absence of cellular infiltration following the association of the nanoparticles to ocular cells. Similarly, NP did not induce inflammatory reactions in the retinal tissue or disturb the organisation of the surrounding ocular tissues of the eye. However, the cytoarchitecture of the outer retina was distorted to a certain degree and the retinal epithelium was disrupted by the histological manipulation itself. Nevertheless, the absence of this layer in some areas seems not to be a typical component of the tissue reaction to particulate carriers, as reported by other works

[103]. In fact, the outer and inner segments of the photoreceptor layer occasionally disappeared in all the eyes receiving the different formulations tested. The increase of the intraocular pressure and miosis, which arose after the intravitreal injection, could be the cause of this abnormality as it also appeared in the control eyes. In fact, ocular decompression before injection reduces this problem as well as temporary loss of vision and reflux of the injection solution [35].

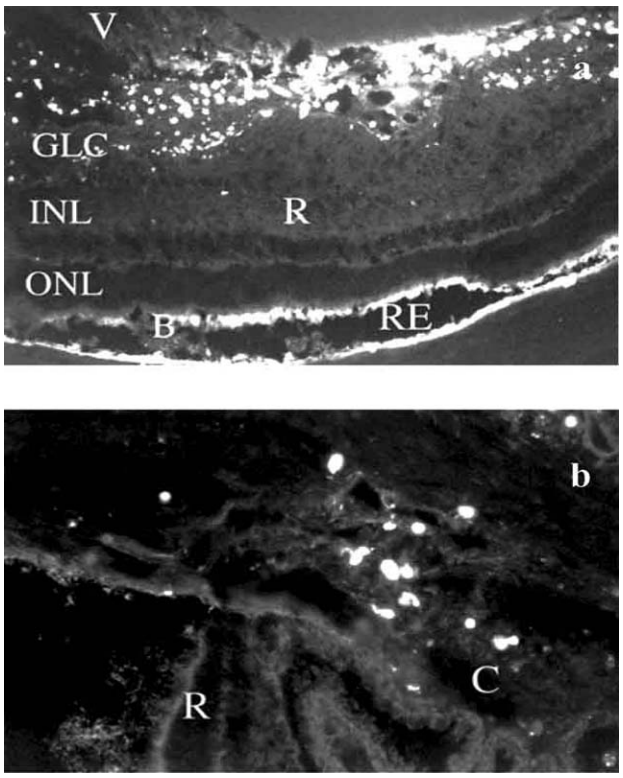

Fig. (9). Disposition of BIO-NP by streptavidin-FITC staining two weeks after their injection in the vitreous cavity (a) and in the blood aqueous barrier adjacent area and ciliary muscle (b). Total magnification: x100 (a) and x200 (b). B: Bacillary layer; C: Ciliary muscle; GLC: Ganglion layer cells; INL: Inner nuclear layer; ONL: Outer nuclear layer; R: Retina; RE: Retinal epithelium; V: Vitreous cavity.

On the other hand, the intravitreal injection may induce the stimulation of pathogenic immune responses, resulting in photoreceptor degeneration, known as "retinitis pigmentosa" [104]. One of the most widely studied pathologies is the experimental autoimmune uveoretinitis (EAU) which leads to considerable damage of the retina and surrounding tissues and which has been extensively studied in rats [105]. Besides the three main identified EUA inducing autoantigens, which are components of retinal photoreceptor cells, there are (i) arrestin [106], (ii) rhodopsin [107,108] and (iii) the inter-photoreceptor binding protein [109]. The staining comparison of these antigens between eyes injected with NP formulations and controls, revealed no differences in their ocular distribution [110]. Thus, these findings enabled us to conclude that the intravitreal administration of NP did not generate organ-specific autoimmune phenomena, which may induce photoreceptor loss.

In summary, albumin nanoparticles appear to be safe carriers for the intravitreal delivery of drugs. They tend to localise mainly in the vitreous cavity-surrounding layer and in areas adjacent to the ciliary body. The retina, as well as other ocular tissues, keeps its cytoarchitecture, with no signs of alteration in the photoreceptor and neuronal layers of the retina. Immunohistochemistry studies also reveal the same expression of two endogenous antigens in ocular tissues when compared to control eyes, confirming the disability of albumin nanoparticles in generating autoimmune phenomena.

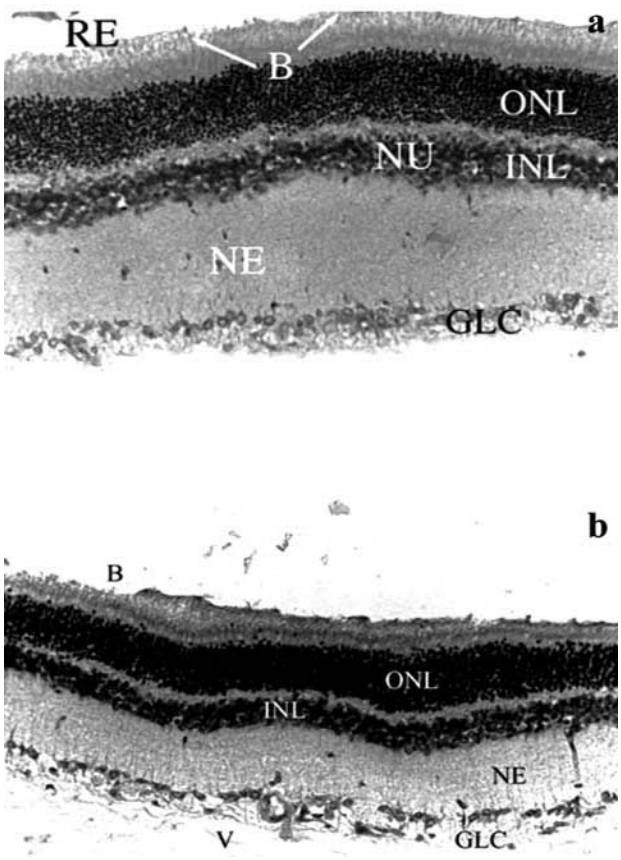

Fig. (10). Histological coloration with hematoxylin-eosin of the retina 2 weeks after injection of empty albumin nanoparticles (a). "Fig. (**10b**)" shows the retina of a control eye injected with saline. Total magnification: x200. B: bacillary layer; GLC: ganglion layer cells; INL: inner nuclear layer; ONL: outer nuclear layer; NE: neuronal interplay layers; NU: inner and outer nuclear layers; RE: retinal epithelium; V: vitreous cavity.

5. CONCLUSION

In the present work, many advantages have been shown for using albumin for preparing nanoparticles: high encapsulation efficiency of various drugs, either as basic as GCV or acidic as the oligonucleotides. Moreover, the conditions for nanoparticle preparation (v.g. step of drug addition, glutaraldehyde concentration) can be modified in order to modulate the release profile in the desirable way. In addition, for antisense therapy, the fusogenic properties of the albumin should be explored more. More importantly, the present work shows an optimal tolerance of these

nanoparticles for intravitreal administration without autoimmune reaction.

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ABBREVIATIONS

AIDS = Acquired immune deficiency syndrome

BIO-NP = Biotin-labelled albumin nanoparticles

CHN = Human corneal fibroblasts

EA = Early antigen expression assay

EAU = Experimental autoimmune uveoretinitis

GCV = Ganciclovir

GCV-NPA = Ganciclovir-loaded albumin nanoparticles obtained by the drug adsorption on the surface of empty nanoparticles

GCV-NPB = Ganciclovir-loaded albumin nanoparticles obtained by incubation of the drug with the protein before the formation of nanoparticles

HCMV = Human cytomegalovirus

MRC-5 = Human embryonic lung fibroblasts

NP = Albumin nanoparticles

PO = 21-Mer phosphodiester oligonucleotide similar to PS

PO-NPA = PO-loaded albumin nanoparticles obtained by the drug adsorption on the surface of empty nanoparticles

PO-NPB = PO-loaded albumin nanoparticles obtained by incubation of the drug with the protein before the formation of nanoparticles

PR = Plaque reduction assay

PS = Fomivirsen

PS-NPA = PS-loaded albumin nanoparticles obtained by the drug adsorption on the surface of empty nanoparticles

PS-NPB = PS-loaded albumin nanoparticles obtained by incubation of the drug with the protein before the formation of nanoparticles

S8D8 = Arrestin

TVS = Transplant vascular sclerosis

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