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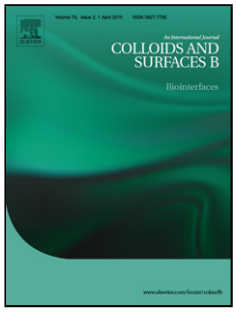

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Gelatin-based nanoparticles as DNA delivery systems: synthesis, physicochemical and biocompatible characterization

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Highlights

. Therapeutic grade DNA molecules requires the escape from the endosomal pathway to the cytosol.

. Gelatin B is an interesting candidate to be used for the effective intracellular delivery.

. pH-triggered release systems have been achieved through the formation of gelatin B (DNA)-PS nanoparticles.

. Gelatin-based nanoparticles as potent and non-toxic intracellular delivery systems.

Abstract

The rapidly rising demand for therapeutic grade DNA molecules requires associated improvements in encapsulation and delivery technologies. One of the challenges for the efficient intracellular delivery of therapeutic biomolecules after their cell internalization by endocytosis is to manipulate the non-productive trafficking from endosomes to lysosomes, where degradation may occur. The combination of the endosomal acidity with the endosomolytic capability of the nanocarrier can increase the intracellular delivery of many drugs, genes and proteins, which, therefore, might enhance their therapeutic efficacy. Among the suitable compounds, the gelification properties of gelatin as well as the strong dependence of gelatin ionization with pH makes this compound an interesting candidate to be used to the

effective intracellular delivery of active biomacromolecules. In the present work, gelatin (either high or low gel strength) and protamine sulfate has been selected to form particles by interaction of oppositely charged compounds. Particles in the absence of DNA (binary system) and in the presence of DNA (ternary system) have been prepared. The physicochemical characterization (particle size, polydispersity index and degree of DNA entrapment) have been evaluated. Cytotoxicity experiments have showed that the isolated systems and the resulting gelatin-based nanoparticles are essentially non-toxic. The pH-dependent hemolysis assay and the response of the nanoparticles co-incubated in buffers at defined pHs that mimic extracellular, early endosomal and late endo-lysosomal environments demonstrated that the nanoparticles tend to destabilize and DNA can be successfully released. It was found that, in addition to the imposed compositions, the gel strength of gelatin is a controlling parameter of the final properties of these nanoparticles. The results indicate that these gelatin-based nanoparticles have excellent properties as highly potent and non-toxic intracellular delivery systems, rendering them promising DNA vehicles to be used as non-viral gene delivery systems.

Keywords: DNA, nanoparticles, DNA entrapment, endosomolytic escape, haemolyisis, *in vitro* cytotoxicity

1. Introduction

The success in the application of nanomedicines and gene therapy is largely dependent on the development of the vectors that can selectively and efficiently deliver the gene or therapeutic agents to the target cells with minimal toxicity [1]. Despite the efforts given in vector technology, development of methods for efficient and protected delivery of therapeutic agents to the target cells still remains a main issue [2]. Owing to the size and charge of naked DNA and the enzymatic and membrane barriers imposed by the cell, the entry of DNA molecules into the cells and subsequent expression represent a very wasteful process [3]. The observation that free plasmid DNA is able to transfect he skeletal muscle [4] the liver [5] or a tumour [6] when given in the appropriate way, but will normally be degraded in the systemic circulation [7], provides the rationale for ‘packaging’ of the DNA. This packaging occurs with the help of a delivery system that tends to compact and protect the nucleic acid [8-11].

The rapidly rising demand for therapeutic grade DNA molecules requires associated improvements in encapsulation and delivery technologies. This includes the formulation of

DNA molecules into synthetic delivery systems for enhanced cellular transformation efficiencies. Research works on colloidal delivery systems in genetic therapeutics are based on the molecular level focusing on the interdisciplinary development of pharmaceutical DNA delivery approaches. Colloidal delivery systems modify many physicochemical properties, aiming to protect the DNA from degradation, minimize DNA loss, prevent harmful side effects, enhance DNA targeting, increase drug bioavailability, and stimulate the immune systems [12-17].

The advances in strategies for treating a wide variety of diseases require an efficient delivery of the active compounds into the cytosol of target cells. One of the challenges for the efficient intracellular delivery of therapeutic biomolecules after their cell internalization by endocytosis is to manipulate the non-productive trafficking from endosomes to lysosomes, where degradation may occur. Endosomes are vesicles formed within a cell during forms of endocytosis in which the material to be ingested first binds to receptor sites on the cell surface. Because the nanocarriers generally cannot directly cross the lipid bilayer of the endosomes, the pH targeting approach, which can lead to a selective disruption of the endosomal membrane, is regarded as a promising strategy to promote a specific triggered release of active biomolecules. The combination of the endosomal acidity with the endosomolytic capability of the nanocarrier can increase the intracellular delivery of many drugs, genes and proteins, which, therefore, might enhance their therapeutic efficacy [18]. The intracellular delivery of active biomacromolecules from endosomes into the cytoplasm generally requires a membrane-disrupting agent. Since endosomes have a slightly acidic pH, pH sensitive compounds could be potentially useful for this purpose since they can destabilize membrane bilayers by pH-triggered conformational change.

Gelatin forms thermally reversible gels with water, and the gel melting temperature (<35°C) is below body temperature, which gives gelatin products unique organoleptic properties and flavour release. Competitive gelling agents like starch, alginate, pectin, agar, carrageenan, etc. are all carbohydrates from vegetable sources, but their gels lack the melt in the mouth, elastic properties of gelatin gels. Gelatin is regarded as a food ingredient rather than an additive and it is Generally Regarded as Safe (GRAS). In 1993 the FDA reiterated the GRAS status of gelatin and stated that there was no objection to the use of gelatin from any source and any country provided that the hide from animals showing signs of neurological disease were excluded and also Specified Raw Materials were excluded from the

manufacturing process [19]. The particularities regarding the use of gelatin as a vehicle to release drugs into cells have been recently reviewed [20]. This review states that gelatin is one of the most versatile natural biopolymers. Due to its biocompatibility, low cost, biodegradability and varied available groups it is widely used in pharmaceutical research for attaching targeting molecules.

The gelification properties of gelatin as well as the strong dependence of gelatin ionization with pH makes this compound an interesting candidate to be used to the effective intracellular delivery of active biomacromolecules. Gelatin B, which pI is 4,8-5,2, is a negatively charged compound at physiological pH. This property might be used to form by interaction with oppositely charged compounds, keeping the protective structure until it enters into the cell. Once the particle enters the cell, where the pH of the endosome would decrease until pH 5, gelatin B becomes positively charged, and due to electrostatic repulsion within positive charges the protection ceases and the entrapped drug can be released into the cell. If appropriated, this hypothesis can be used to the effective intracellular delivery of DNA, RNA or other specific drug [21].

Among the different strategies that can be used to form particles, the interaction between oppositely charged compounds has been selected. In this project, gelatin type B and protamine sulfate has been chosen. Protamine sulfate is protamine from salmon in the sulfate salt (PS), with a molecular mass of 5.1 kDa. Protamines are highly positively charged (overall charge +21), arginine rich proteins that bind to DNA in a non-specific manner via electrostatic interactions. Protamine sulfate adopts random coil conformation in solution. In addition, protein protamine sulfate has been shown to condense DNA [8, 22] and to deliver plasmid DNA into eukaryotic cells [23]. This property, in addition to its longtime use in pharmaceutical formulations, makes protamine a promising candidate in gene delivery formulations. Previous studies in our group has demonstrated the protamine sulfate, alone or in combination with the protein lysozyme, can be used as biocompatible carriers to form DNA gel particles by interfacial diffusion [16, 24]. With regard of the use of protamine in biological systems, it is worth mentioning our recent results, summarising the effect of mixed protein-DNA gel particles systems on the DNA delivery and biocompatibility [16]

In the present work, gelatin (either high or low gel strength) and protamine sulfate has been selected to form particles by interaction of oppositely charged compounds. Particles in the absence of DNA (binary system) and in the presence of DNA (ternary system) have been

prepared. The physicochemical characterization (particle size, polydispersity index and degree of DNA entrapment) have been evaluated as a function of the imposed composition. It is also important to figure out which properties govern the interactions between these nanoparticles and cells. *In vitro* experiments have been performed in order to determine the cytoxicity of the isolated systems and the resulting particles. The feasibility of this concept has been investigated by means of the pH-dependent hemolysis assay and the effect on size and DNA release of the nanoparticles co-incubated in buffers at defined pHs that mimic extracellular, early endosomal and late endo-lysosomal environments.

2. Materials and methods

2.1. Materials

The gelatin from bovine skin (gelatine type B) with gel strength 225 and 75 g Bloom were purchased from Sigma and used as received. The protamine from salmon in the sulfate salt form (PS) with a molecular mass of 5.1 kDa were purchased from Sigma and used as received. The sodium salt of deoxyribonucleic acid (DNA) from salmon testes with an average degree of polymerisation of ≈2000 base pairs (bp) was purchased from Sigma and used as received. The DNA concentrations were determined spectrophotometrically, assuming that for an absorbance of 1 at 260 nm, a solution of dsDNA has a concentration of 50 μg/mL. The absorbance ratios at 260 and 280 nm of the stock solutions were found to be between 1.8 and 1.9, which suggested the absence of proteins. N,N,N',N'-tetramethylacridine-3,6-diamine (acridine orange (AO)) was supplied by Molecular Probes (Invitrogen). 2,5-Diphenyl-3,-(4,5-dimethyl-2-thiazolyl) tetrazolium bromide (MTT), neutral red (NR) dye, and dimethylsulfoxide(DMSO) were obtained from Sigma–Aldrich. Dulbecco´s Modified Eagle´s Medium (DMEM), RPMI 1640 medium, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin–EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA), and penicillin–streptomycin solution(10,000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza. The 75 cm2 flasks and 96-well plates were obtained from TPP. All other reagents were of analytical grade.

2.2. Phase behaviour of gelatins in solution

The phase behaviour of gelation in solution was examined by dissolution of gelatins in PBS buffer (pH 7.4) in a concentration range between 1 and 15 mg/mL. After magnetically

stirring, the resulting solutions were kept overnight in the fridge (10 ºC), in order to promote the gel formation.

2.3. Particle preparation

Protamine sulfate (0.1-10 mg/mL) was dissolved in a PBS buffer (pH 7.4). DNA stock solutions were prepared in 10 mM NaBr to stabilize the DNA secondary structure in its native B-form conformation. In the case of binary systems, gelatin solutions (200 μL) were added dropwise into vigorously gently agitated protein solution (2 mL). Under optimal conditions, droplets from gelatin solutions broken under magnetically stirring and instantaneously gelled into discrete particles on contact with the cationic solution dipped into ice. Thereafter, the particles were equilibrated in the solutions for several periods (0-30 min). After these periods, the confirmation of the particle formation and the size distribution was determined by dynamic light scattering (DLS) at 25 ºC with a Zetasizer Nano ZS90. To measure the particle size distribution of the dispersion, a polydispersity index (PI), ranging from 0,0 for an entirely monodisperse sample to 1,0 for a polydisperse sample, is used. The interpretation of data is performed considering the size distribution by intensity of scattered light.

In the case of ternary systems, mixed gelatin-DNA solutions (200 μL) were added dropwise into vigorously gently agitated protein solution (2 mL). The composition of DNA in the mixed systems was fixed at 50% (v/v) (unless otherwise stated). Under optimal conditions, droplets from the mixed gelatin/DNA solutions broken under magnetically stirring and instantaneously gelled into discrete particles on contact with the cationic solution dipped into ice. The confirmation of the particle formation and the size distribution was undertaken by means of a Zetasizer Nano ZS90, in a similar procedure of that described for particles prepared in the binary system.

2.4. Determination of degree of DNA entrapment

The degree of DNA entrapment in the nanoparticles derived from the ternary system was determined by quantifying the free DNA in the supernatant solution at 260 nm by means of a nanophotomeher (NanoPhotometerTM, Implen). In order to avoid some interference due to the nanoparticles, the nanoparticle dispersions were centrifugated at 10.000 rpm during 30 min. Afterwards, a sample of the supernatant was removed for quantification of the free DNA by spectrophotometry at 260 nm.

The concentration of DNA bound in the DNA gel particles was calculated as the difference between the concentration of DNA added into the cationic solutions and the DNA that remain free in the solutions, after the nanoparticle formation:

[bound DNA]= [total DNA] – [free DNA] (1)

The degree of DNA entrapment is expressed through the loading efficiency values. Loading efficiency (LE) is calculated by comparing the amount of DNA included in the particles with the total amount during particle formation. Loading efficiency (*LE*) were determined by the following equation:

LE (%) = [bound DNA]/ [total DNA] x 100 (2)

Three batches of particles were prepared in each system and the results are given as average and standard deviations.

2.5. Cell cultures

The murine Swiss albino fibroblast, 3T3 and the human epithelial carcinoma, HeLa cell lines were grown in DMEM medium (4.5 g L^{-1} glucose) supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 μg mL^{-1} streptomycin at 37ºC, 5% CO_2. The 3T3 and HeLa were routinely cultured in 75 cm^2 culture flasks and were trypsinised using trypsin-EDTA when the cells reached approximately 80% confluence.

2.6. Cytocompatibility assays

The 3T3 (1 x 10^5 cell mL^{-1}) and HeLa (5 x 10^4 cells $mL^{-1,}$) were seeded into the central 60 wells of a 96-well plate After incubation for 24 h under 5% CO_2 at 37ºC, the spent medium was replaced in the wells with 100 μl of fresh medium supplemented with 5% FBS containing gelatin solutions at the required concentration range (50-2000 μg mL^{-1}). In the case of the nanoparticles derived from the binary or ternary systems, 100 μL of each system diluted 1:1 in fresh medium supplemented with 5% FBS was added in each well.

2.6.1. MTT assay

The MTT assay is based on the protocol first described by Mossman [25]. In this assay, living cells reduce the yellow tetrazolium salt MTT to insoluble purple formazan crystals. After 3T3 and HeLa cell incubation for 24h, the nanoparticles derived from the the binary or

ternary systems or the protein-containing medium were removed and 100 µl of MTT in PBS (5 mg ml^{-1}) diluted 1:10 in medium without FBS and phenol red was then added to the cells. The plates were incubated for a further 3 h, after which the medium was removed. Thereafter, 100 µl of DMSO was added to each well to dissolve the purple formazan product. Plates were then placed in a microtitre-plate shaker for 10 min at room temperature and the absorbance of the resulting solutions was measured at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of tetrazolium salt reduction by viable cells against the untreated cell control (cells with medium only).

2.6.2. NRU assay

Based on the protocol described by Borenfreund and Puerner [26], the NRU assay determines the accumulation of the NR dye in the lysosomes of viable, undamaged cells. Following exposure to the nanoparticles derived from the the binary or ternary systems or the protein-containing medium were removed, and 3T3 and HeLa cells were incubated for 3h with NR dye solution (50 µg ml^{-1}) dissolved in medium without FBS and phenol red. Cells were then washed with PBS, following by the addition of 100 µl of a solution containing 50% ethanol absolute and 1% acetic acid in distilled water to extract the dye. Plates were gently shaken for 10 min to ensure complete dissolution. We then measured the absorbance of the extracted solution at 550 nm using a Bio-Rad 550 microplate reader. The effect of each treatment was calculated as the percentage of uptake of NR dye by lysosomes against the untreated cell control (cells with medium only).

2.7. Interaction with erythrocytes

2.7.1. Preparation of red blood cell suspensions

Rat blood was obtained from anaesthetized animals by cardiac puncture and drawn into tubes containing EDTA. This procedure was approved by the Ethical Committee for Animal Research of the University of Barcelona**.** Serum was removed from the blood by centrifugation at 3,000 rpm (Megafuge 2.0 R Heraeus Instruments) at 4ºC for 10 min, and subsequent suction. The red blood cells were then washed three times at 4ºC by centrifugation at 3,000 rpm with isotonic saline PBS solution, containing 22.2 mmol/L Na_2HPO_4, 5.6 mmol/L KH_2PO_4 and 123.3 mmol/L NaCl in distilled water (pH 7.4). Following the last wash, the cells were diluted to ½ of their volume with isotonic phosphate buffer solution (PBS) (cell density of 8 x 10^9 cell mL^{-1}).

2.7.2.Hemocompatibility and endosomolytic characterization

The membrane-lytic activity of the systems was examined by a hemolysis assay. Red blood cells and gelatin solutions were co-incubated in buffers at defined pHs that mimic extracellular (7.4), early endosomal (6.8), and late endo-lysosomal (< 6.8) environments. A series of different volumes of the gelatin solutions (10 mg mL^{-1}), ranging from 10 to 240 µL, were placed in polystyrene tubes and an aliquot of 25 µL of erythrocyte suspension was added to each tube. The final volume was 1 mL. The tubes were incubated at room temperature for 1 hour under shaking conditions using an Atom 190 shaker (Atom). Following incubation, the tubes were centrifuged (5 min at 10, 000 rpm). The degree of hemolysis was determined by comparing the absorbance (540 nm) (Shimadzu UV-160A) of the supernatant with that of the control samples totally hemolyzed with distilled water. Positive and negative controls were obtained by adding an aliquot of 25 µl of erythrocyte suspension to distilled water and isotonic PBS solution, respectively.

2.8. Effect of environmental acidification on nanoparticles size and DNA release

The effect of pH decrease from 7.4 to 5.0 (mimicking endosomal pH decrease) was investigated by DLS measurements. Particle size measurements of the nanoparticles were performed at 25 °C with a Zetasizer Nano ZS90 (Malvern Instruments, Malvern). The nanoparticulate solutions were titrated from pH 7.4 to pH 5.0 by regularly adding small aliquots of 0.25 M HCl solution.

Simultaneously, the loading efficiency of the nanoparticles at intracellular pH was measured using solutions of nanoparticles prepared at the ternary system. After successive acidification to pH 5.0 by addition of 0.25 M HCl solution and incubation for 1 hour, the nanoparticles were centrifuged at 10,000 rpm for 30 min. Aliquots of supernatant were subsequently was removed for quantification of the free DNA by spectrophotometry at 260 nm by means of a nanophotometer (NanoPhotometerTM, Implen). The percentage of loading efficiency was calculated from the difference of the total amount of DNA added to the solution and the amount of DNA that is determined in the free form in the supernatant.

2.9. Statistical analyses

Experiments were performed at least three times on independent occasions unless otherwise stated. Results are expresses as means standard error of the mean (SEM). Data were

analyzed by PASW Statistics 18 software using one-way analysis of variance (ANOVA) with Scheffé post-hoc tests for multiple comparisons. Each experiment was performed at least three times on independent. Differences were considered statistically significant at $p<0.05$ or $p<0.005$. In the figures significant differences were illustrated with asterisk (unless otherwise mentioned).

3. Results and discussion

3.1. Phase behaviour of gelatins in solution

In order to evaluate the conditions for which the formation of gels of gelatins are favoured, gelatins were dissolved in PBS buffer (pH 7.4) in a concentration range between 1 and 15 mg/mL. The formation of the gels was examined by visual inspection and the stability of them was evaluated indirectly by inversion of the tubs. The formation and stability of the gels seems to be a function of the concentration, gelatin type and the corresponding bloom value. The formation of gels from gelatin type B75 and B225 takes place at concentration up to 11 mg/mL. Regarding their stability, gels from gelatin type B225 demonstrated to be more stable with time than those obtained with the gelatin of lower gel strength (Fig. SM1).

3.2. Particle formation at the gelatin B-protamine sulfate system (binary system)

Once the phase behaviour of gelatins in solution was determined, the formation of nanoparticles at the water-water emulsion-type interfaces by interaction of oppositely charged compounds has carried out. For this purpose, gelatin type B, in its two different gel strength, and protamine sulfate has been chosen. The procedure to the particle formation at the binary systems started with the dropwise addition of a highly viscous gelatin B solution (10 mg/mL) to a protamine solution dipped into ice under stirring to promote the nanoparticle formation. Three different series of experiments have been performed in order to characterize the effect of the agitation time (0 and 30 min) and the ratio between the added gelatin and the protamine sulfate solution (10 and 20 % (v/v)), on the final properties of the obtained nanoparticles. In all cases, the effect of the protamine concentration was evaluated in varying the concentration of this protein from 0.1 to 1.0 mg/mL.

When nanoparticles were prepared at the ratio gelatin type B225/protamine sulfate of 10% (v/v), a general trend can be observed: the size of the obtained particles increased progressively until reach a maximum and then started to decrease (Fig. 1a). This maximum on size corresponds to a protamine concentration equal to 0.5 mg/mL. The observed behaviour suggest that the incorporation of protamine sulfate on the obtained nanoparticles progressively increase until reach the concentration of 0.5 mg/mL. Afterwards, particle size decreased as a consequence of the higher compaction/ condensation degree as the concentration of the cationic agent increased. [16]. This is the general trend observed in the case of nanoparticles prepared with gelatine type B75, independently of the ratio gelatin B75/protamine sulfate (Fig.1c).

When nanoparticles were prepared at the ratio gelatin type B225/protamine sulfate of 20% (v/v), a strong increase on particle size was observed. Although the size decreased as the protamine concentration increased, the higher size obtained under these conditions suggest that the inclusion of protamine sulfate on the particles seems to become more difficult (Fig. 1a). The statistical analysis denotes only significant differences between the three treatments under certain conditions. In addition, when the effect of the gel strength of gelatin on the size and polydispersity index for the same experimental procedure is compared, no significant differences were obtained.

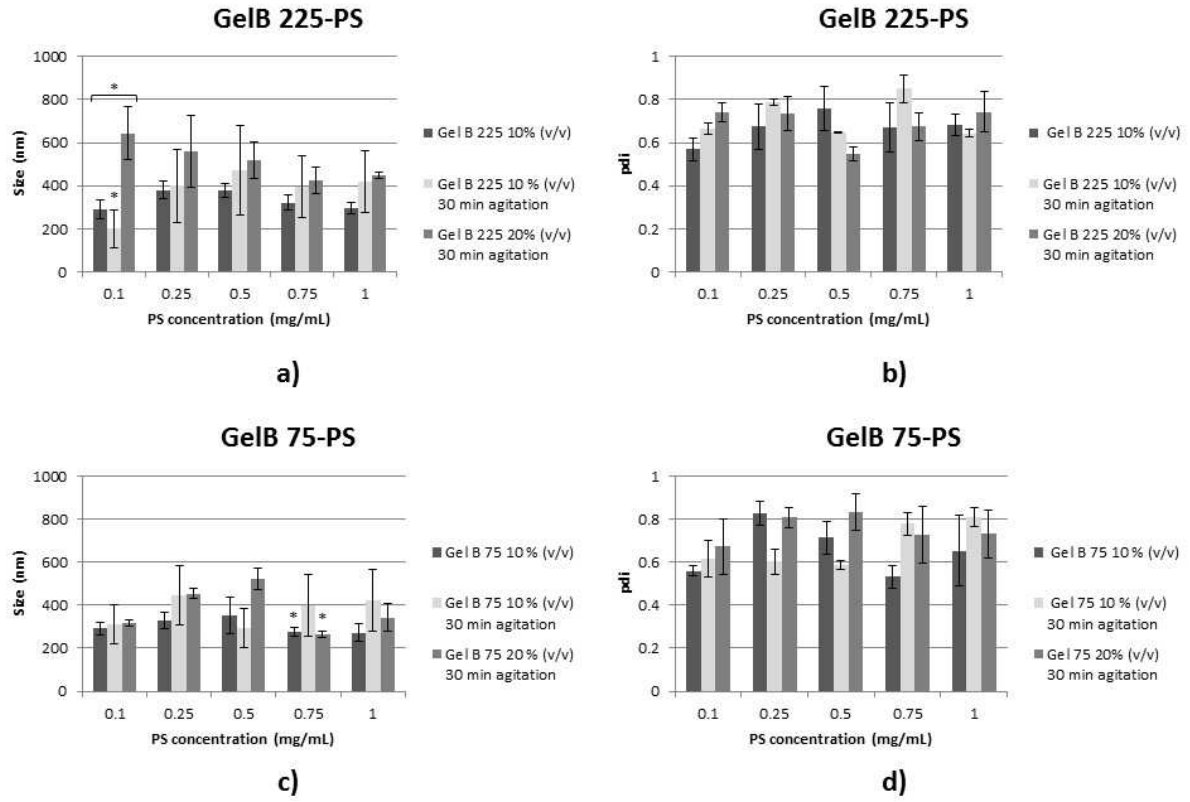


Fig. 1

As an indicator of the stability of the obtained nanoparticles at the binary system, a series of experiments were undergone in the presence of fluorescein as a model of hydrophilic drug. For this purpose, fluorescein was incorporated into the gelatin type B solutions during its preparation. The resulting particles where observed through the fluorescence microscope (Fig. SM2). Under these conditions, only the substances able to emit under a specific wavelength excitation become visible. As shown in the figure, the fluorescent probe appears confined into the particles, confirming the success of the encapsulation process.

3.3. Particle formation at the gelatin B(DNA)-protamine sulfate system (ternary system)

Once the stability of the particles resulting from the gelatin type B-protamine sulfate system was confirmed, as well as their ability to encapsulate substances such as fluorescein, the preparation of the particles at the gelatin B(DNA)-protamine sulfate systems were performed. One of the main objectives in this new series of experiments consisted in determining the maximum amount of DNA that can be incorporated in the gelatin type B solutions, preserving the homogeneity of the solutions. For this purpose, mixed solutions

containing gelatin type B at a concentration equal to 10 mg/mL in PBS buffer (pH 7.4) and DNA at a concentration equal to 10 mg/mL in NaBr 10 mM solution were prepared. The composition of DNA in the mixed systems varied between 10 and 50% (v/v). Fluorescence microscopy using the fluorescence dye acridine orange (AO) was used to confirm the presence of DNA and to assess the secondary structure of the nucleic acid in the mixed solution. AO (excitation: 500 nm/emission: 526 nm) intercalates into double-stranded DNA as a monomer, whereas it binds to single-stranded DNA as an aggregate. On excitation, the monomeric acridine orange bound to double-stranded DNA fluoresces green, with an emission maximum at 530 nm. The aggregated acridine orange on single-stranded DNA fluoresces red, with emission at about 640 nm [27]. When the mixed solutions were examined by means of a transilluminator, fluorescence emission can be observed in the solutions, mainly in those systems in which the composition of DNA is up to 30% (v/v) (Fig. SM3 a). For lower DNA content (10 and 20% DNA (v/v)) it can be observed that DNA is not homogenously distributed on the gelatin type B solution. For DNA content between 40 and 50% (v/v) homogeneous mixed gelatin/DNA systems can be obtained. Due to the homogeneity of the mixed systems as well as the stability of the obtained gels (Fig. SM3 b), the ration gelatin type B/DNA equal to 50% (v/v) was selected to perform subsequent experiments.

The preparation of the nanoparticles at the ternary system was performed under the selected conditions. In a similar way of that performed at the binary system, the ratio gelatin type B225(DNA)/protamine sulfate was equal to 10% (v/v) and the protamine concentration was varying from 0.1 to 1.0 mg/mL. It is noteworthy that once the mixed solutions gelatin type B/DNA came in contact with the protamine sulfate solutions, the formation of precipitates of big dimensions has been observed. It is known that interactions between oppositely charged compounds in aqueous solutions can lead to associative phase separation, where the concentrated phase assumes the form of a viscous liquid, gel, or precipitate. This approach was the basis for developing novel DNA-based materials, including DNA gel particles [17]. The presence of these precipitates, which were not observed in the case of the binary system gelatin type B -protamine sulfate, suggests that the interaction between DNA and protamine sulfate is more favoured.

Although these results confirmed the formation of particles at the gelatin type B (DNA)-protamine sulfate system, the size of the resulting particles were far away of the required

dimensions to be applied for the intracellular delivery of active biomacromolecules. A progressive decrease on the concentration of both gelatin type B and DNA was performed in order to obtain almost colorless solutions confirming the generation of particles at the nanometric scale. Gelatin type B concentration was set at 0.25 mg/mL on varying the DNA concentration on the range between 0.125 and 1 mg/ml. For comparative purposes, particles in the absence of DNA (binary system) were also prepared. The effect of protamine sulfate on the final properties of the obtained particles was evaluated by setting the protein concentration at 0.1 and 1.0 mg/mL. As can be observed in Figure 2a, the presence and concentration of DNA had no influence on the size of particles obtained at the gelatin type B(DNA)-PS0.1 system. The obtained particles displayed a size around 250 nm. The effect of the gelatin gel strength can be also considered negligible. However, the polydispersity index revealed the presence of DNA on the particles. The maximum pdI value corresponds to the particles prepared in the absence of DNA. Moreover, the pdI values decreased almost progressively when the DNA content increased. Significant differences between particles prepared at intermediate and high DNA concentrations and particles prepared in the absence of DNA were found (Fig. 2b). No effect of the gelatin gel strength has been observed.

A different performance was observed when the concentration of protamine sulfate was increased ten times. Under these conditions, the size of the obtained particles increased progressively when the DNA content increased. Significant differences between particles prepared at intermediate DNA concentrations and particles prepared in the absence of DNA were found (Figure 2c). The size varied between 100 nm, in the absence of DNA, and 500-600 nm for the gelatin typeB(0.75DNA)-PS1.0 systems. These results can be correlated with an increase on the protamine sulfate inclusion on the particles. For the highest DNA content, a decrease on the size is observed. This reduction on size can be correlated with an increase on the compaction/ condensation degree between the oppositely charged compounds DNA and protamine sulfate. Only under few experimental conditions significant differences between the gelatin gel strength were observed.

In the present work, the observed pdI values resulted to be a function of the gelatin gel strength, DNA and PS concentration (see Fig. 2). However, the absence of presence of DNA on the formulations seems to be a controlling parameter of the PDI values. Nanoparticles prepared in absence of DNA demonstrated to show the highest pdI values (> 0.8) for both gelatin gel strength and both PS concentrations (Fig. 2 c and Fig. 2d). The results suggested

that under these conditions, a broad polydisperse distribution of nanoparticles have been obtained. However, in the presence of DNA, pdI values can be modulated from 0.2 to 0.8 by variation of the initial DNA concentration. In most cases, significant differences between PDI values corresponding to nanoparticles in the presence and absence of DNA have been found, as indicated with a symbol mark ($^{\ddagger}p < 0.05$ and $^{\#}p < 0.005$). Consequently, the initial DNA concentration on the formulations contributes positively to the moderate polydisperse distribution of the obtained nanoparticles. In addition, when the effect of the protamine sulfate concentration on the size and polydispersity index obtained for the same experimental conditions (DNA concentration and gelatin gel strength) was compared, significant differences were obtained only under certain conditions (Figure 2e and 2f).

By looking at the values of the polydispersity and size as a function of the PS/DNA concentration ratio, the optimal size with low polydispersity can be found. Using the data showed in Fig 2 is possible to deduct that for particles obtained at the gelatin type B(DNA)-PS0.1, values of PS/DNA about 0.1-0.5 give the low polydispersity close to 0.2 while other values for the PS/DNA result in particles with high polydispersity. Consequently, DNA concentrations ranged between 0.5 and 1 mg/mL can be considered as the optimal DNA concentration values in terms of particle size and polydispersity values.

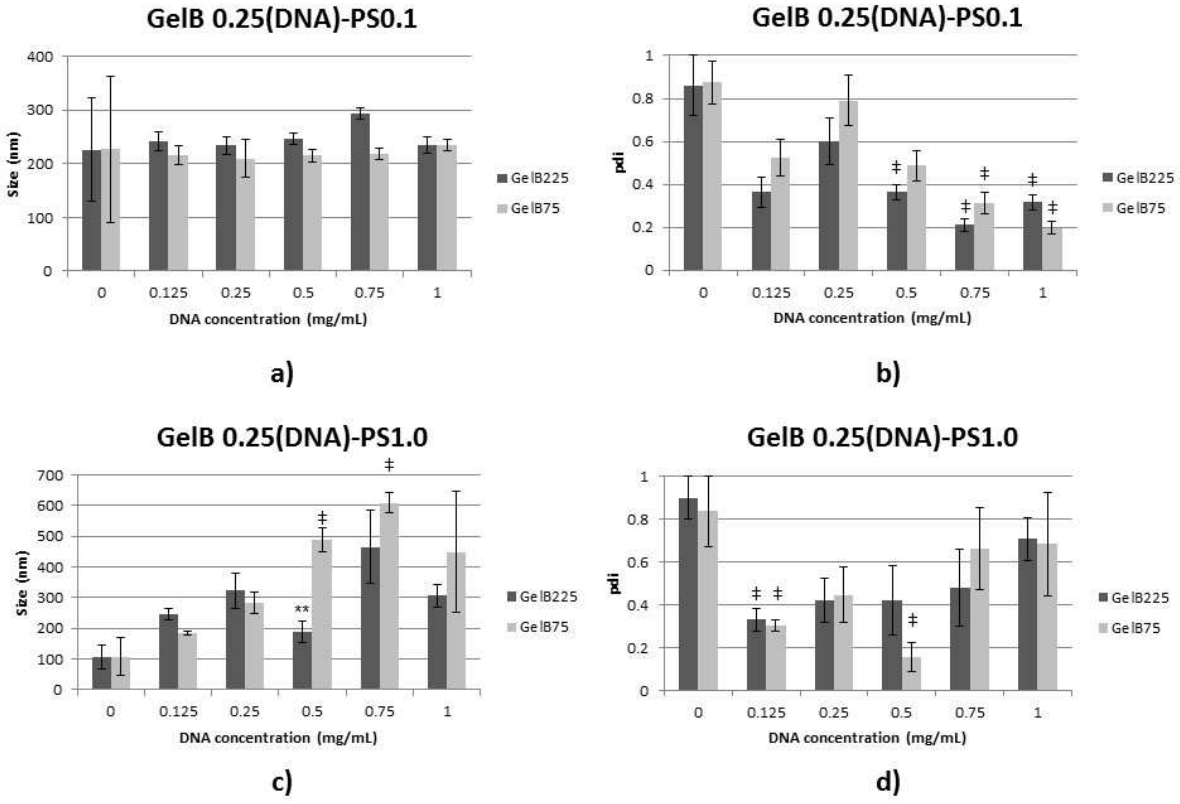
The particle suspensions were investigated by TEM techniques without further purification. Although using this procedure most of the particles can appear embedded in the corresponding protein solution, it is possible to distinguish individual particles (indicated with black arrows, for cryoTEM and by white arrows, for negative staining TEM, Figure SM1). The sizes of the resulting nanoparticles are in good agreement with the experimental data determined by DLS techniques.

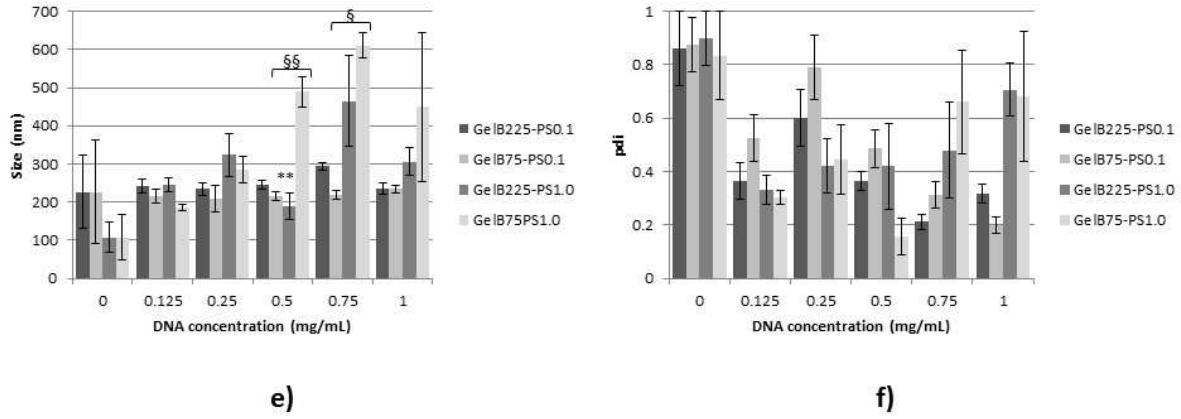
3.4. Degree of DNA entrapment on the gelatin B(DNA)-protamine sulfate nanoparticles

The success of the encapsulation process was evaluated as a basis of the DNA entrapment on the gel B (DNA)-PS nanoparticles. The degree of DNA entrapment can be expressed as a function of the loading efficiency (LE) values. LE measures the amount of DNA that is included in the particles with respect to the total DNA, during particle formation.

Fig. 3a and 3b summarize the characteristics of the nanoparticles obtained at the ternary. LE values ranged between 64 and 98% have been obtained for systems using protamine sulfate concentrations equal to 1.0 mg/mL. Interestingly, LE values reacted in a similar fashion when

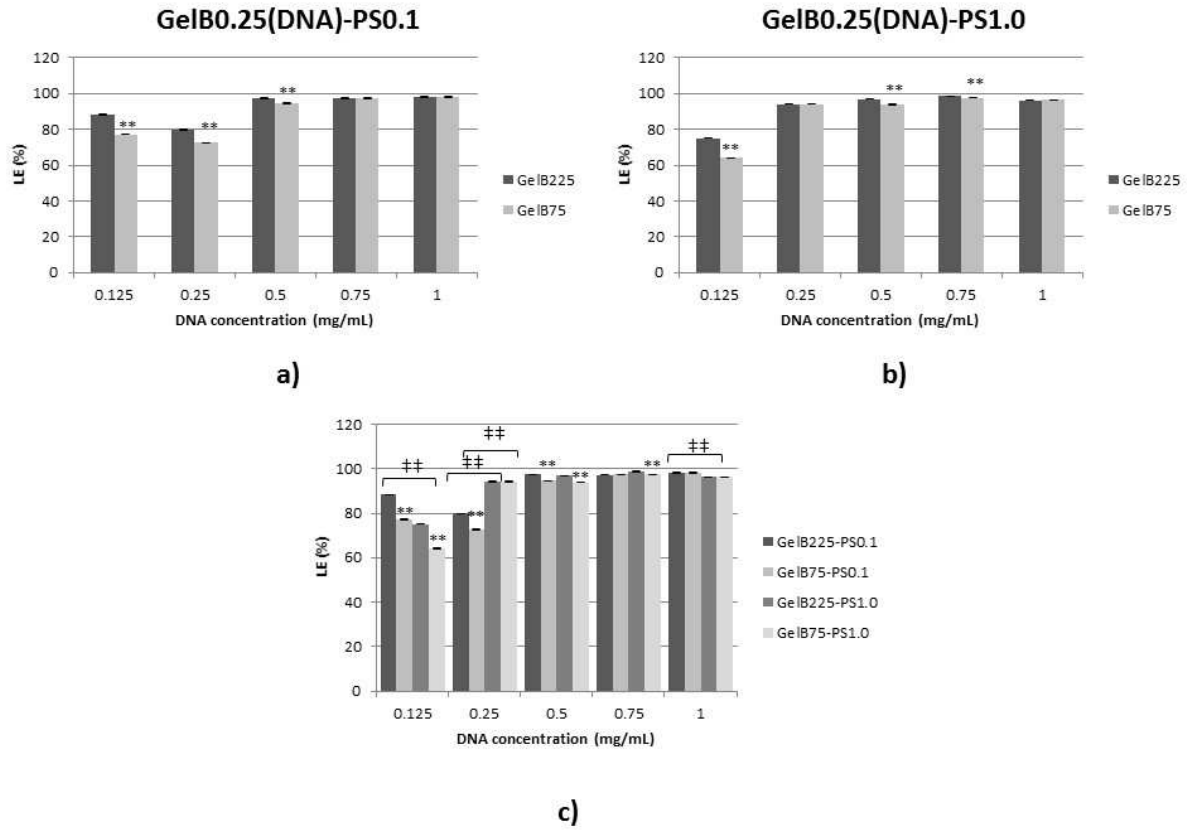
the protamine sulfate concentration was reduced ten times (LE ranged between 72 and 98%). The obtained LE values confirm the effectiveness of DNA entrapment under these conditions. When the effect of gelatin gel strength was evaluated for the same protamine sulfate concentration, significant differences between these two blooms values were obtained. The obtained results suggested that DNA entrapment is favoured when gelatin with the higher gel strength was used. When the effect of protamine sulfate for the same experimental conditions was compared, significant differences can be obtained: a protamine sulfate concentration equal to 0.1 mg/mL promoted higher LE values for the lowest and the highest DNA concentrations values; for protamine concentration equal to 1.0 mg/mL, higher LE values are obtained for discrete conditions (DNA concentration equal to 0.25 mg/mL) (Fig. 3c).





e) f)

Fig. 2.



a) b)

c)

Fig. 3.

3.5. Cytotoxicity in vitro

Cytotoxicity plays a critical role in the efficiency of the delivery vectors. In order to deliver the DNA into the cells, the cationic particles bind to the cell surface by electrostatic interaction, promote endocytosis and release the genetic material inside the cell. Unfortunately, while high concentrations of the delivery agents imply an increased chance of the DNA penetrating the cell nucleus, they can also interfere with physiological processes within the cell, inducing cell death. Thus, present research is aimed at designing gene delivery agents that are able to deliver DNA into the cells with minimal toxicity [28].

The interaction of the obtained nanoparticles and their component was determined with non-tumor (3T3 fibroblast) and tumor (HeLa) cell lines. Assessing the capacity of live cells to metabolize a tetrazoliumcolorless salt to a blue formazan (MTT assay) as a measurement of cell metabolic activity within the mitrocondrial compartment, and the diffusion through intact cell membranes to accumulate within lysosomes (NRU assay) were used to perform indirect measurements of cell viability.

The first approach on the determination of the cytotoxic response was performed with gelatins in solutions. Dose–response curves for each gelatin, determined by MTT and NRU assays using tumor cell line HeLa and non-tumor cell line 3T3 fibroblasts, are given in Fig. SM4. The cytotoxicity assays were performed in the concentration range 50 and 2000 μg/ mL. Gelatins showed low cytotoxicity towards HeLa cells, which displayed viability in the range 77% to 100% and 84 and 100%, as determined by the MTT and NRU assays, respectively. Moreover, when the results obtained between the two different endpoints were compared, it can be deduced that the MTT assay was more sensitive in detecting the cytotoxic effects within the four studied gelatins than the NRU assay did. Similar results were obtained in the case of 3T3 cell line. Due to the high viability observed at the tested protein concentrated range, it was not possible to define the protein concentration required to inhibit cell growth by 50% compared with an untreated control (IC_{50}). Consequently, this value can be defined as higher than 2000 ug/mL for the two studied gelatins. The statistical analysis denotes only significant differences between the two endpoint points under certain conditions.

Previous studies carried out in our group have revealed that, as with other cationic derivatives, protamine sulfate displayed concentration-dependent toxicity towards cells *in vitro* [16]. PS showed cell viabilities ranged from 7% to 100%, depending on the concentration. These experiments enabled us to define the protein concentration required to inhibit cell growth by 50% compared with an untreated control (IC_{50}). For PS, it was found to

be 140 and 250 μg/mL for 3T3 and HeLa cell lines, respectively. Concerning DNA/protamine complexes, studies performed with other authors demonstrated that those complexes have little cytotoxicity [29]. Consequently, the putative cytotoxic response of the gelatin-based nanoparticles can be derived from the contribution of the protamine sulfate.

Once the relative viability of the isolated components was determined, the cytotoxic effect of the nanoparticles obtained at the ternary systems was evaluated. Taking into account the obtained results concerning the particle size, the degree of DNA entrapment as well as the demonstrated cytotoxicity of protamine sulfate, the cytotoxic study was restricted to the ternary sytems prepared at a protamine concentration equal to 0.1 mg/mL. For comparative purposes, particles in the absence of DNA (binary system) were also tested. Fig. 4 summarizes the obtained results as a function of the cell line and endpoint assay.

A general trend in the cytotoxicity responses of the nanoparticles prepared at the ternary system is that the cell viabilities provided by these systems showed lower values that those observed by nanoparticles obtained in the absence of DNA (binary systems). Although the molecular details of the mechanism by which cationic carriers mediate DNA delivery are still poorly understood, current evidence supports the hypothesis that the DNA complexes enter cells by means of endocytosis. The efficiency of cellular uptake and subsequent intracellular processing, a prerequisite for effective cellular transfection, may well depend on particle size. In this work, the physicochemical characterization of the obtained particles, including their size distribution was performed (Figure 2). Although the nanoparticles prepared in the absence of DNA showed higher polydispersity than the DNA-containing nanoparticles, both series of nanoparticles were obtained in a size range for which significant differences have not observed.

The observed differences on cell viability as a function of the DNA concentration seem to be a function of both the cell line and the endpoint assays. When 3T3 fibroblasts were used, significant differences between particles prepared in presence and absence of DNA were found, by the MTT assay (Figure 4a). The cell viabilities varied between 45 and 75 % as a function of DNA and gelatin gel strength. Remarkably, cell viabilities ranged between 100 and 160 % were observed using the NRU assay (Figure 4b). No significant differences between particles prepared in presence and absence of DNA were found were observed. When HeLa cell line was used, significant differences between particles prepared in presence and absence of DNA were found, by both the MTT and NRU assay (Figure 4c and 4d). The

cell viabilities varied between 45 and 100 % for MTT and 17 and 100% for NRU, as a function of DNA and gelatin gel strength. Interestingly, the studied endpoints assays appeared to be selective to the deleterious effect of nanoparticles prepared with different gelatine gel strength. Whereas MTT assay seems to be more sensitive to the effects of nanoparticles prepared with gelatin with the lower gel strength value, NRU discriminated better between the effects of nanoparticles prepared with gelatin with the higher gel strength.

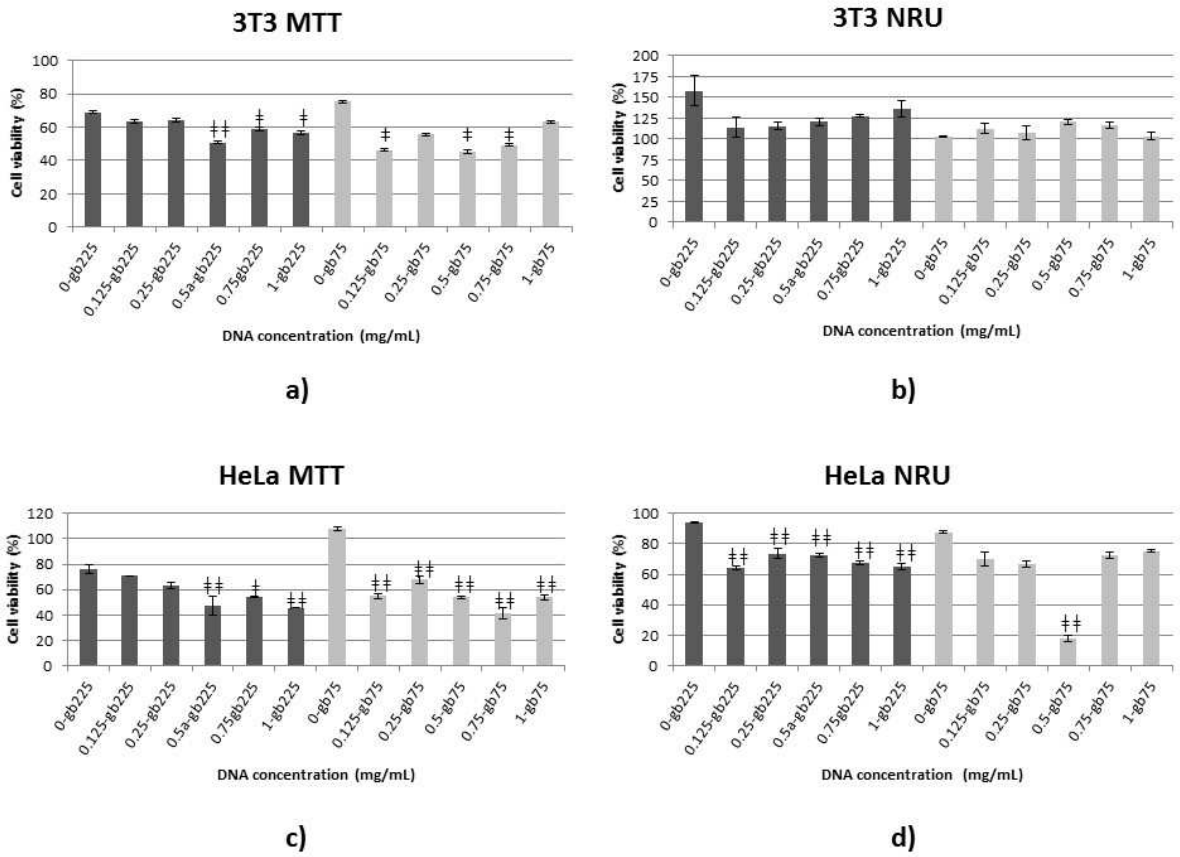


Fig. 4.

In addition, the cytotoxic properties of the obtained nanoparticles were evaluated in order to establish selective responses as a function of cell line type and endpoint method (Fig. 5). When the effect of the endpoint method was evaluated in 3T3 cell line (Fig.5a), greater cytotoxicity have been detected by MTT than by NRU endpoint. It is well established that the MTT assay is a measurement of cell metabolic activity within the mitochondrial

compartment, while NRU assay measure membrane integrity. NR dye diffuses through intact cell membranes to accumulate within lysosomes [30, 31]. Based on the mechanisms of cell damage detected by each cytotoxicity assay, the obtained results suggested that the toxicity mechanism of nanoparticles obtained at the binary and ternary systems involve an earlier interaction with the mitochondrial compartment while the plasma membrane and/or lysosomal compartments could be affected at a later stage. Under these conditions, almost in all cases there are significant differences between the obtained values by MTT and NRU methods ($p<0.05$ and $p<0.005$). No significant differences were found when the effect of gel strength on the cytotoxic responses of nanoparticles prepared under the same experimental conditions was compared.

In contrast, more significant differences between nanoparticles prepared with gelatin with different gel strength were observed with Hela cell line (Fig.5b). Together with some significant differences on the cell viabilities observed as a function of the endpoint method, for which MTT seems to promote higher cytotoxic responses, differences between the cytotoxic responses promoted by nanoparticles prepared with different gelatin gel strength could be establish under discrete conditions. In the case of nanoparticles prepared in the absence of DNA (binary system), higher cytotoxic responses were detected for the formulations containing the gelatin with higher gel strength, especially with the MTT method. In contrast, higher cytotoxic responses were observed in the case of the nanoparticles prepared with the lower gelatin gel strength at the DNA concentration of 0.5 mg/mL determined by the NRU method.

When the results obtained between the two different endpoints were compared, it could be deduced that, in general, the NRU assay gave higher cell viabilities than the MTT assay did (Fig.5c and 5d). However, the latter assay was more sensitive in detecting the effect of gelatin gel strength on particles prepared in the absence of DNA (binary systems) (Fig. 5c). These results suggest that the nanoparticles prepared with gelatin with higher gel strength have a greater effect on the metabolic activity than on plasma membrane on the cells, especially in the case of the HeLa cell line. In contrast, by NRU methods, HeLa cells seem to be more sensitive to the deleterious effect of nanoparticles prepared in presence of DNA (0.5 mg/mL of DNA) and gelatin with the lower gel strength. Therefore it can be concluded that the tumoral cell line HeLa seems to be more sensitive to the effect of gelatin gel strength, presence or absence of DNA on the particles and endpoint method.

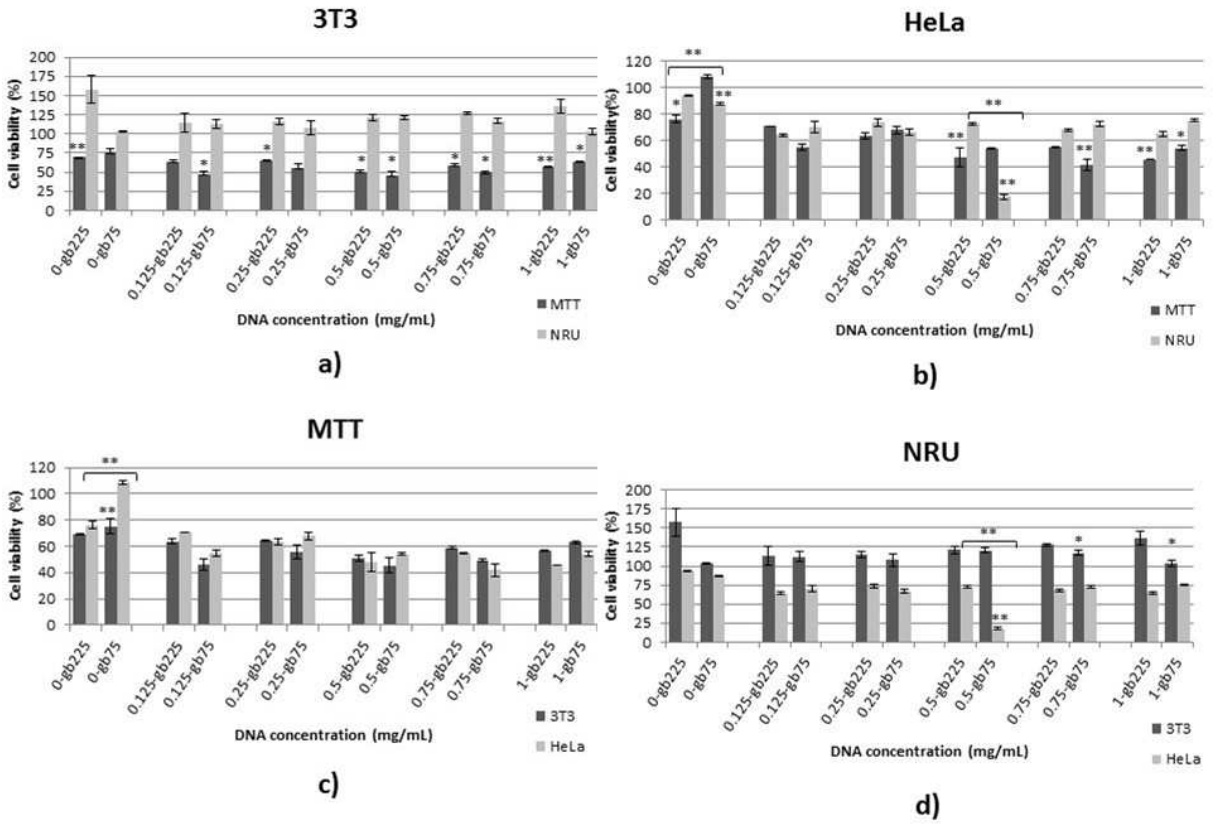


Fig. 5..

3.6. Hemocompatibility and endosomolytic characterization

One useful model system for screening endosomolytic behavior is the *ex vivo* pH-dependent hemolysis assay. In this model system, the erythrocyte membrane served as a surrogate for the lipid bilayer membrane that encloses endolysosomal vesicles. This generalizable model has been used by others to evaluate the endosomolytic behavior of cell-penetrating peptides and other polymeric gene delivery systems [32-35].

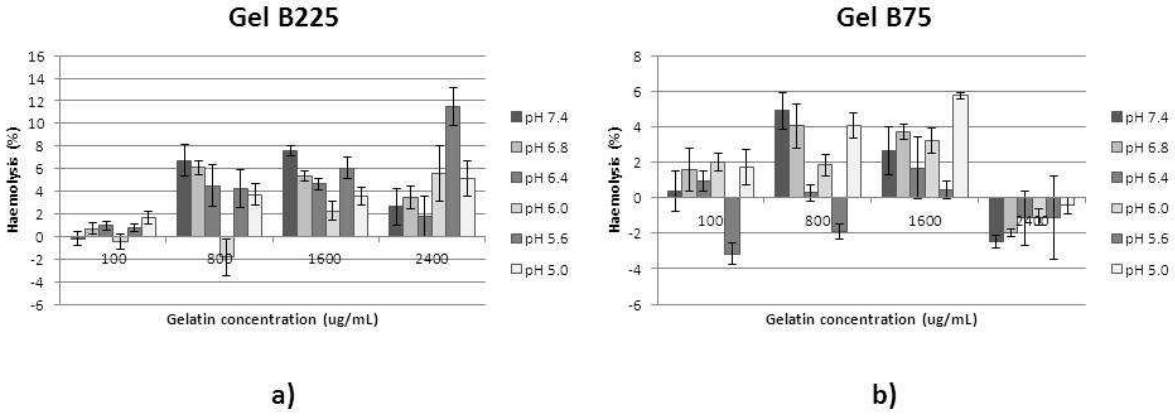
In this protocol, red blood cells and test materials were co-incubated in buffers at defined pHs that mimic extracellular (7.4), early endosomal (6.8), and late endo-lysosomal (< 6.8) environments. From screening a small library of potentially endosomolytic test materials, one can infer that samples that produce no hemolysis at pH 7.4, but significantly elevated hemolysis at pH < 6.5, will be the most effective and cytocompatible candidates for cytosolic drug delivery. Materials that fit these criteria would be expected to remain inert and not indiscriminately destroy lipid bilayer membranes until being e6xposed to a drop in the local pH following internalization into endolysosomal compartments [36].

Red blood cells were co-incubated at pH 5.0, 5.6, 6.0, 6.4, 6.8 and 7.4 with gelatin type B225 and gelatin type B75 at the range of concentration from 100 to 2400 μg/mL (Fig.6). In these experiments two types of information can be derived. First of all, the haemolytic potential of a material can be defined as the measure of the extent of haemolysis that may be caused by the system when it comes into contact with blood. Accordingly, the results obtained at pH 7.4 make possible the evaluation of the haemolytic character of these two gelatins. From a haemolytic point of view, values lower than 5% can be considered a permissible level [37]. Taking into account the obtained results, gelatin type B225 seems to be slightly haemolytic at concentrations of 800 and 1600 μg/m. However, the haemolytic values obtained with gelatin type B75 were always lower than the permissible level.

The haemolysis assay could be also used as rapid screen for endosomolytic activity of intracellular drug delivery systems. In the hemolysis assay, red blood cells and gelatins were incubated in PBS buffer at defined pH that mimics extracellular, early endosomal, and late endolysosomal enviroments. In this model system the erythrocyte membrane serves as a surrogate for the lipid bilayer membrane that encloses endolysosoma vesicles. Typically, ideal endosomolytic agents exhibit dose-dependent and pH-dependent hemolytic behavior. Figure 7a shows the hemolytic responses of gelatin type B225 at the selected pH values. Under the studied conditions, gelatin type B225 showed a concentration-dependent haemolysis. However, concerning the pH dependence, it seems to be a function of the gelatin concentration. If for 100 and 2400 μg/mL, the haemolytic response increase as the pH value decrease, for intermediate concentration, the opposite trend could be observed. The gel strength of the gelatin derivative also plays an important role on the assignment of endosomolytic activity though the haemolysis assay. When the effect of pH on the haemolytic response of red blood cells to gelatin type B has been determined, the effect of both concentration and pH is observable. Concerning the later, the hemolysis responses seem to perform in the expected way for concentrations equal to 100 and 1600 μg/mL, being the opposite at 800 μg/mL.

Interestingly, at the highest studied concentration, negative haemolysis values were obtained. These results, lacking in meaning concerning haemolysis,were in connection with the fact that gelatins can form gels, for which adsorption of the media can be expected. In the light of these considerations, it seems that at the highest studied concentration, gelatin type B75 was able to adsorb the haemoglobin released into the media by effect of the gelatin, in

comparison with the untreated erythrocytes control (erythrocytes only with medium at the corresponding pH values). The obtained results demonstrated that the higher the pH the greater the adsorption is. These results can be correlated with the ionization state of haemoglobin as a function of pH. Human haemoglobin shows a pI around 7.1 [38]. Assuming that this value would be similar in the case of haemoglobin derived from erythrocytes from rat, the released haemoglobin would present a positive charge in almost all the studied pH interval. Gelatin type B, however, would present a negative charge until pH values close to 5, for which the change of ionization state would occurs. Consequently, there is a pH interval for which haemoglobin and gelatin type B presented opposite charges, favouring the electrostatic interaction between them and increasing the adsorption. For pH at values close to pH 5, both proteins would present positive charges, minimizing the electrostatic interaction between them and, consequently, modulating the adsorption. Statistical analyses didn't denote significant differences between the haemolytic results at different pH values.



a) b)

Fig. **6.**

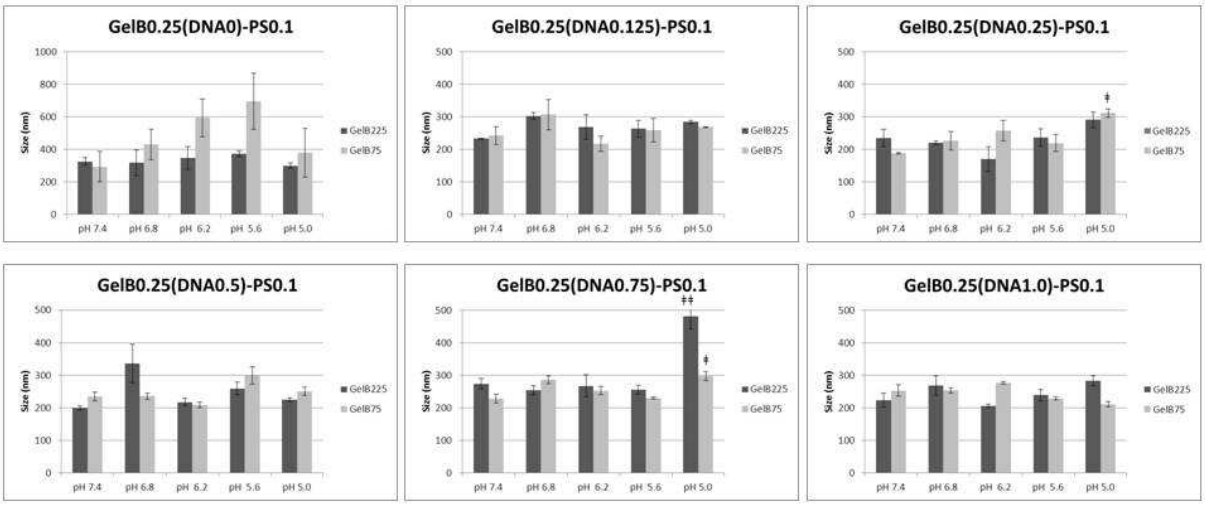
3.7. Effect of environmental acidification on nanoparticles size and DNA release

Since electrostatic interactions between the negatively-charged gelatin type B and the positively-charged protamine sulfate are the main driving force for the self-assembly into the nanoparticles, it can be anticipated that a decrease in pH, as is occurring during endosomal

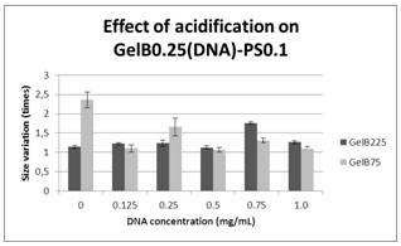
acidification, would influence the stability of the nanoparticles. A decrease of the pH would also lead to destabilization and eventually release of DNA from the nanoparticles prepared at the ternary system [39].

DLS measurements showed that the gelatin type B75-PS nanoparticles significantly increase in size from ca. 300 nm to 700 nm when the pH decreases from 7.4 (extracellular pH) to 5.0 (intracellular pH). In contrast, no effect was observed in the case of gelatin type B225-PS nanoparticles (Figure 7a). These results are in the light of that observed when the endosomolytic response was determined by means of the haemolysis assay (Fig. 6). In comparison, the corresponding DNA-containing nanoparticles slightly responded to pH decrease. In general, a discrete increase on size of less than 100 nm could be observed by acidification from extracellular to intracellular pH values. This result may be attributed to the strong interaction between DNA and protamine sulphate, as was already described. Significative differences between size values for pH 7.4 and pH 5.0 were found in discrete cases (Figure 7a). The final size variation between extracellular and intracellular pH as a function of the initial DNA concentration is summarized in Fig. 7b.

The DNA release from the gelatin type B (DNA)-PS nanoparticles by acidification from pH 7.4 (extracellular pH) to pH 5.0 (intracellular pH) is summarized in Fig. 8. The DNA release upon successive acidification seems to be a function of both the gelatin gel strength as well as the initial DNA concentration. A general trend is that the DNA released from nanoparticles increased progressively for pH values corresponding to early to late endosomes. However, in the case of nanoparticles prepared with gelatin type B225 the DNA released seems to start to pH values corresponding to early endosomes (pH 6.8). The percentage of DNA released result to be a function of both the gelatin gel strength and the initial DNA concentration. The percentage of DNA released decreased exponentially as a function of the initial DNA concentration, with percentages of DNA released ranged between 50 to 15 % , and 40 and 10%, for nanoparticles prepared with gelatin type B225 and gelatin type B75, respectively (Fig. 8b). The maximum amount of DNA released from the nanoparticles is 12 and 17 µg/mL for nanoparticles containing gelatin type B75 and gelatin type B225, respectively (Fig. 8c).



a)



b)

Fig. 7

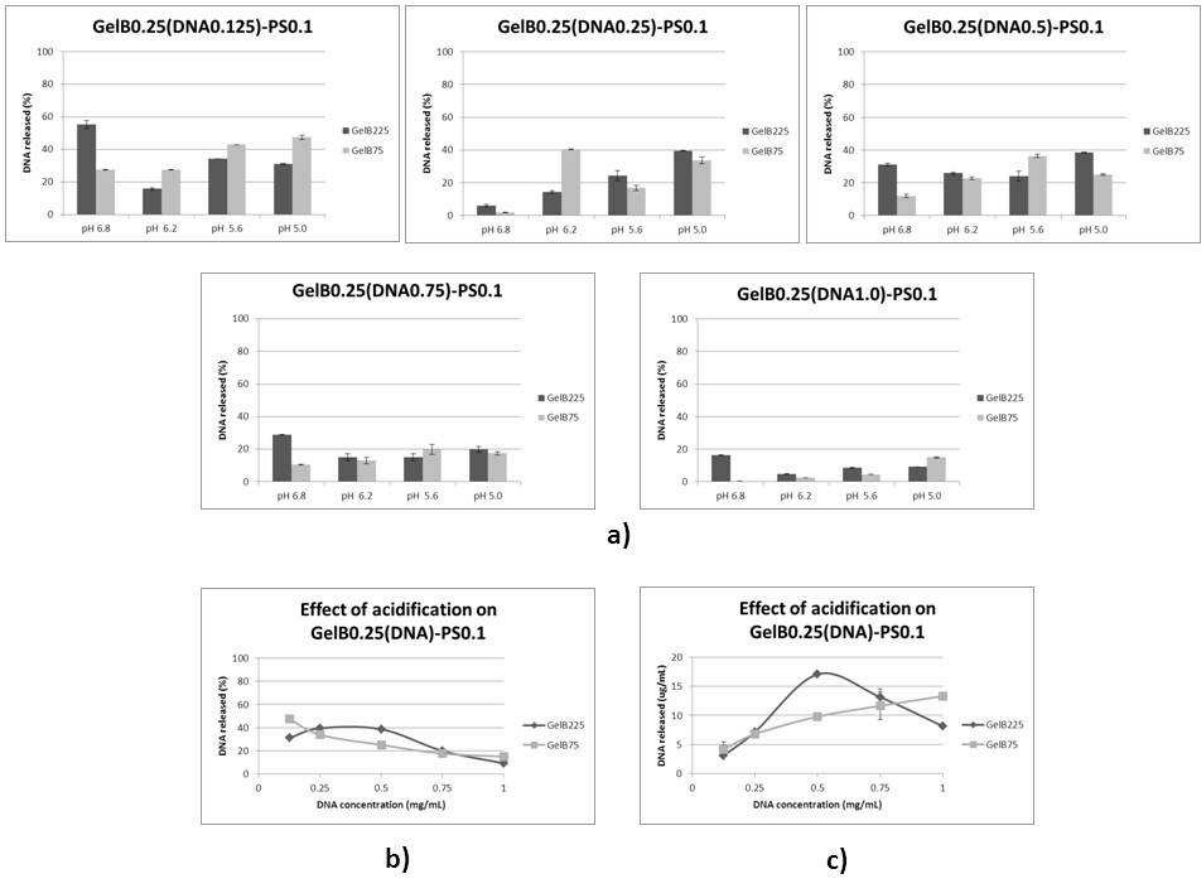


Fig. 8

4. Conclusions

The interaction between oppositely charged compounds has been the basis for developing gelatin-based nanoparticles. By mixing solutions of gelatin type B (either high or low gel strength) with solutions of protamine sulfate the formation of nanoparticles with sizes around 400 nm but high polydispersity has been obtained. .As a first requirement for systems to function as intracellular delivery vectors is that they should be able to condense their content into nanosized particles. Both the size (around 200 nm) and the polydispersity were strongly decreased when DNA was included on the nanoparticles formulations (ternary systems). DNA was effectively entrapped on the gelatin B(DNA)-protamine sulfate nanoparticles with LE values ranged between 72 and 98%, confirming the effectiveness of the encapsulation process.

Cytotoxicity plays a critical role in the efficiency of the delivery vectors. The interaction of the obtained nanoparticles and their component was determined with non-tumor (3T3 fibroblast) and tumor (HeLa) cell lines, using MTT and NRU assays to perform indirect

measurements of cell viability. Whereas gelatin type B resulted to be non-cytotoxic in nature, with IC_{50} values higher than 2000 ug /mL, the putative cytotoxicity of the obtained nanoparticles seems to be a function of the inclusion of protamine sulfate. A general trend in the cytotoxicity responses of the nanoparticles prepared at the ternary system is that the cell viabilities provided by these systems showed lower values that those observed by nanoparticles obtained in the absence of DNA (binary systems). The cytotoxic properties of the obtained nanoparticles were evaluated in order to establish selective responses as a function of cell line type and endpoint method. It can be concluded that the tumoral cell line HeLa seems to be more sensitive to the effect of gelatin gel strength, presence or absence of DNA on the particles and endpoint method.

The hemolysis assay has been demonstrated to be a useful model for screening the endosomolytic behaviour of gelatins in solution. By co-incubation of red blood cells with gelatin solutions in buffers at defined pHs that mimic extracellular and intracellular environments, the effect of pH has been evaluated. Gelatins resulted to be non-haemolytic compounds with haemolysis values lower than 5 % at physiological pH. When the nanoparticles were inserted in buffer solutions that reaches pH endosomal values (pH ~5), different responses have been encountered: particle destabilization, size modification and DNA release. Upon acidification of the solution, as occurs after endosomal uptake, the gelatin B(DNA)-protamine sulfate nanoparticles tend to destabilize as is evidenced from their observed increase in size in DLS experiments. DNA can be effectively released, with maximum percentages ranged between 40 and 50% DNA released, as a function of both the gelatin gel strength and the initial DNA concentration. The results indicate that these gelatin-based nanoparticles have excellent properties as highly potent and non-toxic intracellular delivery systems, rendering them promising DNA vehicles to be used as non-viral gene delivery systems. Current studies are focused on determining the mechanism of nanoparticles uptake and internalization.

Acknowledgements

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FIGURE CAPTIONS

Fig. **1.** Effect of protamine sulfate concentration and preparation method on the size and polydispersity index, respectively of nanoparticles obtained with gelatin type B225 (a,b) and gelatin type B75 (c,d). The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; *$p < 0.05$ denotes significant differences.

Fig. **2.** Effect of DNA concentration and bloom strength on the size and polydispersity index, respectively of nanoparticles obtained with protamine sulfate at 0.1 mg/mL (a,b) and protamine sulfate at 1.0 mg/mL (c,d). Effect of protamine sulfate concentration on the size (e) and polydispersity index (f) of nanoparticles prepared under the same DNA concentration and gelatin bloom strength. The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; ‡$p < 0.05$ denotes significant differences regarding particles formed in the absence of DNA (binary systems), **$p < 0.005$ denotes significant differences regarding particles formed under the same experimental conditions with different gelatin gel strength, and $^{\S}p<0.05$ and $^{\S\S}p<0.005$ denotes significant differences regarding particles formed under the same experimental conditions with different protamine sulfate content.

Fig. **3.** Effect of DNA concentration and bloom strength on the loading efficiency (LE) values of nanoparticles obtained with protamine sulfate at 0.1 mg/mL (a) and protamine sulfate at 1.0 mg/mL(b). Effect of protamine concentration on the loading efficiency (LE) values of nanoparticles obtained for the same DNA concentration and bloom strength (c). The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; **$p < 0.05$ denotes significant differences between different gelatin gel strength and $^{\#}p <0.005$ denotes significant differences between different protamine sulfate concentration.

Fig. **4.** Effect of DNA concentration and bloom strength on the relative viabilities of 3T3 (a, b) and HeLa (c, d) cells treated with the different nanoparticles during 24h determined by MTT and NRU assays, respectively. The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test, ♮p < 0.05 and ♯p < 0.005 denotes significant differences regarding particles prepared in the absence of DNA (binary systems).

Fig. **5.** Cell viabilities on 3T3 (a) and HeLa (b) cell lines on varying the endpoint method, and cell viabilities determined by MTT (c) and NRU (d) methods on varying the cell line type. The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test; *p < 0.05 and **p < 0.005 denotes significant differences.

Fig. **6.** Effect of pH and concentration on the haemolytic responses of gelatin type B225 (a) and gelatin type B75 (b). The data correspond to the average of three independent experiments ± standard deviation.

Fig. **7.** Effect of acidification from pH 7.4 (extracellular pH) to pH 5.0 (intracellular pH) on the size of the nanoparticles prepared in absence (binary systems) and presence of DNA (ternary systems) (a). The data correspond to the average of three independent experiments ± standard deviation. Statistical analyses were performed using ANOVA followed by Scheffé's multiple comparison test, ♮p < 0.05 and ♯p < 0.005 denotes significant differences regarding size values obtained a pH 7.4 (extracellular pH). Size variation (times) from pH 7.4 (extracellular pH) to pH 5.0 (intracellular pH) as a function of the initial DNA concentration (b). The data correspond to the average of three independent experiments ± standard deviation.

Fig. 8. Effect of acidification from pH 7.4 (extracellular pH) to pH 5.0 (intracellular pH) on the release of DNA from nanoparticles (a). The data correspond to the average of three independent experiments ± standard deviation. Percentage of DNA released from the nanoparticles from pH 7.4 (extracellular pH) to pH 5.0 (intracellular pH) as a function of the initial DNA concentration (b). Amount of DNA released from the nanoparticles from pH 7.4 (extracellular pH) to pH 5.0 (intracellular pH) as a function of the initial DNA concentration (c). The data correspond to the average of three independent experiments ± standard deviation.