

## Research Article

# Combining Hyaluronic Acid with Chitosan Enhances Gene Delivery

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The low gene transfer efficiency of chitosan-DNA polyplexes is a consequence of their high stability and consequent slow DNA release. The incorporation of an anionic polymer is believed to loosen chitosan interactions with DNA and thus promote higher transfection efficiencies. In this work, several formulations of chitosan-DNA polyplexes incorporating hyaluronic acid were prepared and characterized for their gene transfection efficiency on both HEK293 and retinal pigment epithelial cells. The different polyplex formulations showed morphology, size, and charge compatible with a role in gene delivery. The incorporation of hyaluronic acid rendered the formulations less stable, as was the goal, but it did not affect the loading and protection of the DNA. Compared with chitosan alone, the transfection efficiency had a 4-fold improvement, which was attributed to the presence of hyaluronic acid. Overall, our hybrid chitosan-hyaluronic acid polyplexes showed a significant improvement of the efficiency of chitosan-based nonviral vectors *in vitro*, suggesting that this strategy can further improve the transfection efficiency of nonviral vectors.

## 1. Introduction

Nonviral gene therapy is currently limited by the lack of vectors with gene transfer efficiency similar to viral vectors. Chitosan is one of the most studied cationic polymers for nonviral gene therapy, both *in vitro* and *in vivo* [1–3]. Although chitosan-based polyplexes (complexes of chitosan-nucleic acids) have desirable characteristics for gene therapy such as efficient nucleic acid encapsulation and protection against degradation, they show low gene transfer efficiency, which is the major obstacle to its use as a gene therapy vector [1, 4]. Several studies suggest a direct correlation between the stability of polyplexes and transfection efficiency, proposing that the high stability and strong interactions between chitosan and DNA are the cause for the low transfection results [2, 5, 6]. Polyplex stability is thus a crucial parameter when

designing a polymer based gene delivery vector; it should be stable enough to withstand the cellular internalization process, but not too stable that once inside the cell it will not release its therapeutic load [7, 8].

Approaches for improving the efficiency of chitosan-mediated gene transfer focus on chemical modification of chitosan and the incorporation of anionic biopolymers. We have previously modified chitosan to incorporate disulfide bonds that could be cleaved intracellularly, but the increase in transfection efficiency was moderate [9]. The incorporation of anionic polymers, which destabilize polyplexes and hence facilitate DNA release [4, 7, 8, 10, 11], is another strategy that has shown promising results. Competition-binding assays showed that the addition of alginate effectively reduces the interaction strength between CS and DNA, which improved DNA release and consequently transfection

[10]. Other studies have shown that coating polyplexes with hyaluronic acid (HA) enhanced internalization and transfection in association with other cationic polymers, such as polyethyleneimine [12, 13]. It has also been previously shown that the incorporation of HA into the polyplex formulation increased green fluorescent protein (GFP) expression and the authors suggest this increase to be related to (1) improved internalization due to interactions with the cellular surface, (2) HA function as a transcription activator, and (3) HA accumulation in the perinuclear region and cell nuclei [14]. Furthermore, several studies hypothesized that HA could be used to improve targeting through the specific HA/CD44 receptor interaction and could be of value to both gene and drug delivery strategies [14, 15].

In this work, we designed CS polyplexes incorporating HA (CSHA) with two different molecular weights (MW) in several CS to HA ratios and evaluated their potential *in vitro* for gene delivery. While other studies have used CSHA polyplexes targeting the anterior part of the eye [12, 14, 16], this is, to our knowledge, the first report in the posterior part of the eye. We have tested these formulations in cells of the retinal pigment epithelium, a cell layer of the retina that supports the overall health of the retina [17] and whose function, when compromised, is implicated in several retinal pathologies such as age-related macular degeneration, among others.

## 2. Materials and Methods

**2.1. Materials.** Chitosan (CS) with a MW of 15 kDa and a degree of deacetylation of 84% was purchased from Polysciences Inc., USA. Hyaluronic acid, with 132 or 214 kDa, from here on designated HA132 or HA214, respectively, was purchased from Lifecore Biomedical Inc., USA. All other reagents were of analytical grade and were used without further purification.

**2.1.1. Plasmid and Cell Lines.** A plasmid expressing enhanced green fluorescent protein (GFP) driven by the cytomegalovirus promoter (kindly provided by Dr. Jean Bennett, University of Pennsylvania, USA) was amplified in Top 10 bacteria and purified using a Plasmid Maxi kit (Qiagen, USA) according to the manufacturer's instructions. Plasmid DNA (pDNA) was dissolved in TE buffer, and the concentration was determined at 260 nm using a NanoDrop 2000c spectrophotometer (Thermo Scientific, USA).

Two cell lines were used in the cytotoxicity and transfection studies: human embryonic kidney 293 (HEK293) cells, a cell line generally used to assess transfection efficiency, and human retinal pigment epithelial cells (ARPE-19), a cell line derived from normal human eyes. All cell culture reagents were purchased from Sigma-Aldrich (USA).

### 2.2. Methods

**2.2.1. Preparation of CS and CSHA Polyplexes.** CS polyplexes were prepared as previously described by our lab [9]. Briefly, a CS solution (0.02% (W/V) in 0.1 M acetic acid, pH 3) and

a 5 mM of sodium sulphate solution were separately heated to 55°C. Equal volumes of both solutions were quickly mixed together, vortexed for 30 s, placed on ice, and stored at 4°C.

CSHA polyplexes were prepared using a HA132 or HA214 solution (0.1% (W/V) in MilliQ water). The following CS : HA weight ratios: 3 : 1, 4 : 1, 5 : 1, 7 : 1, and 10 : 1, were used, keeping constant the CS amount (50 µg) and varying the HA amount. In order to use equal volumes of both solutions, HA was diluted in 5 mM sodium sulphate and then mixed with the CS solution, as described above.

To prepare CS and CSHA polyplexes loaded with pDNA at a  $\text{NH}_3^+ : \text{PO}_4^-$  ratio of 5 : 1, 50 µg of CS and 16.1 µg of pDNA were used. pDNA was mixed with the sodium sulfate solution and this solution was mixed with the CS solution, as described above, thus producing CSpDNA 5 : 1 or CSHApDNA 5 : 1 polyplexes. The 5 : 1 ratio was chosen based on previous results for CS-pDNA, which showed this ratio to be the most appropriate for cell transfection [1, 9].

**2.2.2. Polyplex Characterization.** Size analysis was performed using dynamic light scattering (DLS) and noninvasive backscatter technology with a detection angle of 173°, and zeta potential (ZP) was measured using laser Doppler velocimetry and phase analysis light scattering technology (Zetasizer Nano ZS, Malvern instruments, UK). All polyplexes were analyzed in ddH<sub>2</sub>O at 25°C. The polydispersity index (PdI) was calculated based on DLS measurements using the Zetasizer Nano Series software v 6.20.

Transmission electron microscopy (TEM, JEOL JEM-1011 electron microscope, Tokyo, Japan) was used to evaluate the morphology of the polyplexes. Prior to analysis, samples were stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar films.

**2.2.3. Evaluation of pDNA Encapsulation Efficiency and Protection from Degradation.** The pDNA complexation, retention, and integrity in the polyplexes were assessed by gel electrophoresis. Free pDNA and polyplexes were separately incubated with 1 unit of DNase I (Sigma-Aldrich, USA) for 15 min at 37°C. The reaction was stopped by 1 µL of a 50 mM EDTA solution and heated at 70°C for 10 min. The integrity of the pDNA was then analyzed by agarose gel electrophoresis in 1% (W/V) agarose in TAE buffer with ethidium bromide. Gels were subjected to a 70 mV voltage for 1.5 h and further visualized under UV light (AlphaImager, Alpha Innotech, USA).

**2.2.4. Evaluation of Polyplex Stability.** Polyplex stability was evaluated in two different ways: stability at physiological temperature (37°C) and pH (7.4) and also long-term stability (4°C, pH 7.4). Briefly, polyplexes were incubated in equal volumes of either PBS or DMEM (with 10% FBS [fetal bovine serum]) at 37°C for 1 to 3 days. Polyplex stability, evaluated by pDNA retention, was performed as described in the previous section. For evaluation of long-term stability, polyplexes were incubated at 4 or 37°C and their size and polydispersity monitored weekly for up to 23 weeks.

TABLE 1: Composition, size, polydispersity, and zeta potential of CS and CSHA polyplexes.

Particle	pDNA <sup>§</sup>	HA (kDa)	CS : HA ratio	Size ( $\varnothing$ , nm)	Polydispersity	Zeta potential (mV)
CS	–	—	—	402.8 $\pm$ 94.10	0.273 $\pm$ 0.128	19.9 $\pm$ 2.38
CSpDNA	+	—	—	241.8 $\pm$ 64.04 <sup>****</sup>	0.266 $\pm$ 0.068	20.09 $\pm$ 2.28
CSHA1	–	—	5 : 1	351.3 $\pm$ 24.79 <sup>#</sup>	0.245 $\pm$ 0.092	23.57 $\pm$ 1.19
CSHA2	+	132	—	328.1 $\pm$ 50.25	0.260 $\pm$ 0.088	24.18 $\pm$ 3.06
CSHA3	–	—	7 : 1	334.7 $\pm$ 62.76 <sup>#</sup>	0.157 $\pm$ 0.066	18.20 $\pm$ 2.30
CSHA4	+	—	—	267.7 $\pm$ 30.52 <sup>****</sup>	0.293 $\pm$ 0.080	19.08 $\pm$ 3.56
CSHA5	–	—	5 : 1	360.5 $\pm$ 69.66 <sup>#</sup>	0.183 $\pm$ 0.064	24.29 $\pm$ 3.19
CSHA6	+	214	—	322.5 $\pm$ 69.94	0.270 $\pm$ 0.097	23.04 $\pm$ 2.31
CSHA7	–	—	7 : 1	345.6 $\pm$ 43.05 <sup>#</sup>	0.243 $\pm$ 0.068	20.84 $\pm$ 4.48
CSHA8	+	—	—	284.9 $\pm$ 34.31 <sup>****</sup>	0.298 $\pm$ 0.065	20.00 $\pm$ 2.60

<sup>§</sup>Presence or absence of pDNA in the formulation is indicated by + or –, respectively. Values marked with asterisks are statistically different to CS value, \*\*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . Values marked with cardinals are statistically different to CSDNA value, # $P < 0.05$ , ## $P < 0.01$  (statistical differences determined by Tukey's multiple comparisons test).

**2.2.5. Cell Culture.** Cells were cultured at 37°C, under a 5% CO<sub>2</sub> atmosphere. Different culture media were used, according to each cell's specifications: HEK293 in Dulbecco's Modified Eagle's Medium (DMEM) and ARPE-19 in DMEM mixture with F-12 HAM, both supplemented with 10% of fetal bovine serum, 1% penicillin/streptomycin, and 1% Glutamine.

**2.2.6. Cytotoxicity Evaluation.** An MTT assay was performed to evaluate the cytotoxicity of the polyplexes. Cells were plated at a density of  $1.5 \times 10^4$  cells/well in 48-well flat bottom tissue culture plates and the assay was carried out as described previously [9]. Cells were incubated in culture medium containing different amounts of polyplexes (from 0.667 up to 13.3  $\mu$ g of CS per cm<sup>2</sup> of growth area) for 72 h. As positive and negative controls of cell viability, cells cultured in standard cell culture conditions and cells incubated with a latex extract in culture medium (1.5 cm<sup>2</sup>/mL) were used, respectively. Absorbance was measured using a microplate reader (Tecan Infinite 2000, USA), at 570 and 630 nm, for cell viability/formazan formation and background, respectively. After subtracting the background (OD = OD<sub>570 nm</sub> – OD<sub>630 nm</sub>), cell viability was calculated as follows: cell viability (%) = (OD<sub>sample</sub>)/(OD<sub>control</sub>)  $\times$  100, where OD<sub>control</sub> and OD<sub>sample</sub> are cells not challenged and challenged by polyplexes, respectively. Each value was averaged from triplicates and each experiment was carried out thrice.

**2.2.7. Transfection Studies.** For the transfection studies, cells were plated at  $1 \times 10^5$  cells/well in 6-well tissue culture plates. FuGENE HD (Promega, USA) was used as positive transfection control according to the manufacturer's instructions. CS and CSHA polyplexes were added to plated cells at a ratio of 1  $\mu$ g of pDNA per well and further incubated for 72 h. Nontransfected cells were used as negative transfection control.

Transfection efficiency was evaluated quantitatively by flow cytometry by scoring GFP positive cells (FACScalibur, BD Biosciences, USA) using FL-1H, green channel. A total of  $1 \times 10^5$  events were counted for each sample. The percentage

of positive events corresponds to the gated events minus the nontransfected cells.

**2.2.8. Statistical Analysis.** Results presented are mean  $\pm$  standard deviation of at least three independent experiments. Statistical analysis was performed with GraphPad Prism 5 software. Data were subjected to analysis of variance (one- and two-way ANOVA) and multiple comparisons tests using a confidence interval of 95%.

### 3. Results

**3.1. Size, Polydispersity, Surface Charge, and Morphology.** The initial step in polyplex characterization was the determination of polyplexes size, polydispersity (PdI), and surface charge (by zeta potential, ZP), and the results are presented in Table 1. For some of the tested CS : HA ratios (3 : 1, 4 : 1, and 10 : 1) we observed aggregation and consequently large sizes and high PdI. These ratios were not tested further (data not shown).

Polyplexes formulated without pDNA had sizes between 334.7  $\pm$  62.76 nm and 402.8  $\pm$  94.10 nm while those formulated with pDNA revealed sizes between 241.8  $\pm$  64.04 nm and 328.1  $\pm$  50.25 nm. The observed decrease in size is statistically different between CS polyplexes and pDNA loaded polyplexes CSDNA, CSHA4, and CSHA8; differences were also found between CSDNA polyplexes and pDNA unloaded CSHA formulations.

All mean PdI values are under 0.300, reflecting homogeneous preparations. The only statistically significant differences found were between formulations CSHA3 and CSHA4 and CSHA3 and CSHA8 ( $P \leq 0.05$ ). These might reflect minor differences in the homogeneity of the polyplex suspensions mainly due to the presence of an additional anionic compound in the mixture. Polyplexes were produced as a single homogeneous preparation as illustrated by the DSL size distribution graphs (Figure 1), where no secondary peaks were observed. Regarding the surface charge of the polyplexes, all displayed a positive charge with ZP values between 18.20  $\pm$  2.30 and 24.29  $\pm$  3.19. The ZP values that were

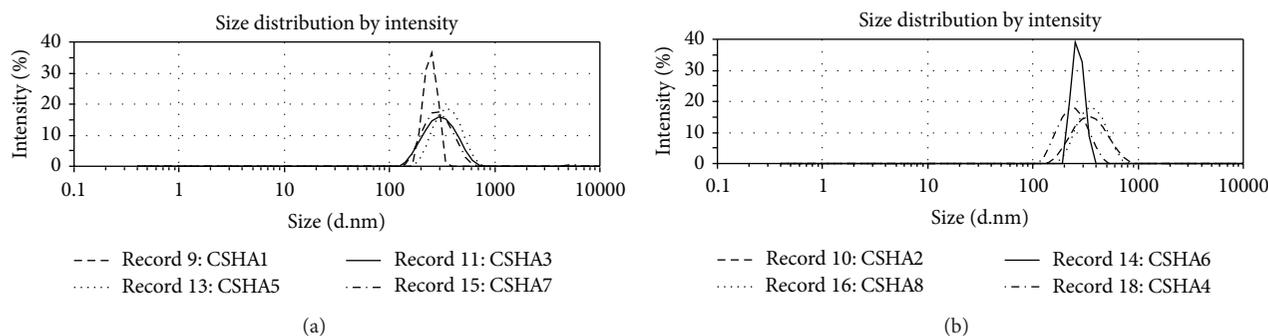


FIGURE 1: Dynamic light scattering raw data representative graphs for CSHA polyplexes: (a) without pDNA and (b) with pDNA.

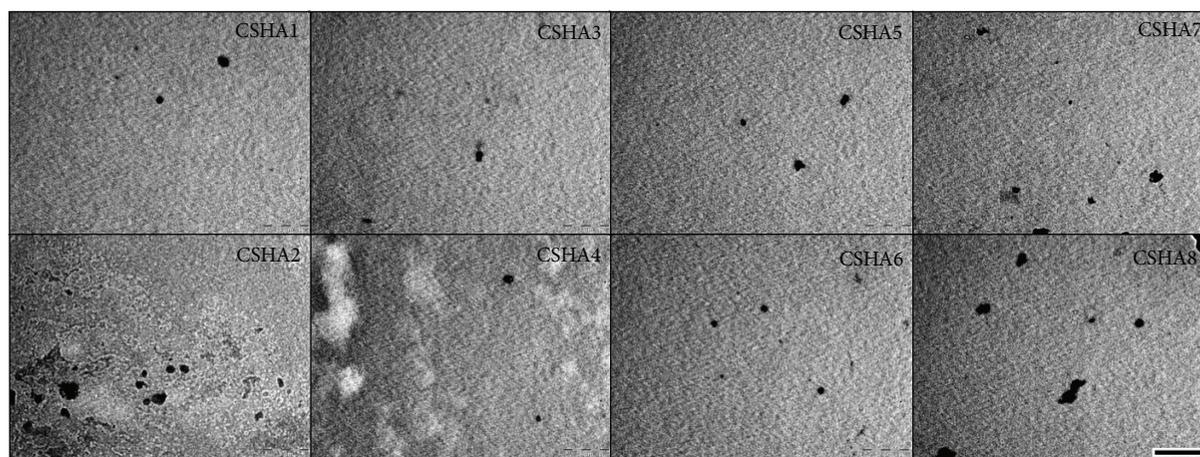


FIGURE 2: TEM microphotographs of CSHA polyplexes showing that different formulations have a regular, close to spherical morphology (amplification: 100,000x, scale bar represents 2  $\mu\text{m}$ ).

found to be statistically different were only for CSHA2 versus CSHA3 and CSHA3 versus CSHA5 ( $P \leq 0.05$ ).

The morphology of the polyplexes was also analyzed by TEM and the microphotographs revealed near spherical polyplexes with sizes consistent to the ones determined by DSL (Figure 2).

**3.2. pDNA Complexation and Protection from DNase Degradation.** The second step in the characterization of the polyplexes was to evaluate if the presence of HA in the different formulations affected pDNA complexation and protection against nuclease degradation. All formulations complexed pDNA successfully, as observed by the absence of pDNA migration in an agarose gel (Figures 3(a) and 3(b)). Polyplexes without pDNA (CS and CSHA) were used as controls and, as expected, did not produce any detectable signal. These results were further confirmed by performing the same experiment in a 0.3% agarose gel (Figure 4).

Regarding pDNA protection against nuclease degradation, all formulations showed a similar behavior, with no detectable pDNA degradation (Figures 3(c) and 3(d)). Uncomplexed pDNA was used as a control and, as expected, was completely degraded by DNase I.

**3.3. Polyplex Stability.** Stability at physiological conditions is an important factor since polyplexes should be stable enough to protect pDNA and only release it once it has reached the intracellular milieu. We therefore tested the stability of the polyplexes at physiological pH and in the presence of serum, to mimic *in vivo* conditions, by incubating the polyplexes with PBS (pH 7.4) or DMEM with 10% FBS, respectively. No detectable pDNA release was observed regardless of the formulation and period of incubation (Figure 5).

The shelf life of the produced polyplexes is also an important factor to consider. Long-term stability of the polyplexes was evaluated at 4 and 37°C by analysis of their size and PdI (Figure 6). Results depicted in Figure 6 were evaluated by variance analysis (1-way ANOVA) and Dunnett's post hoc test to compare values to the starting time point ( $t = 0$ ). All CSHA formulations performed very similarly and therefore the results shown for formulations CSHA7 and 8 are representative of the other CSHA formulations.

The results showed that both size and PdI directly increased with temperature and that all tested formulations remained stable at 4°C for an extended period of time (up to 13 weeks). The size of CSDNA polyplexes remained constant throughout time. The presence of pDNA in the formulation seemed to increase stability in CS formulations. Although

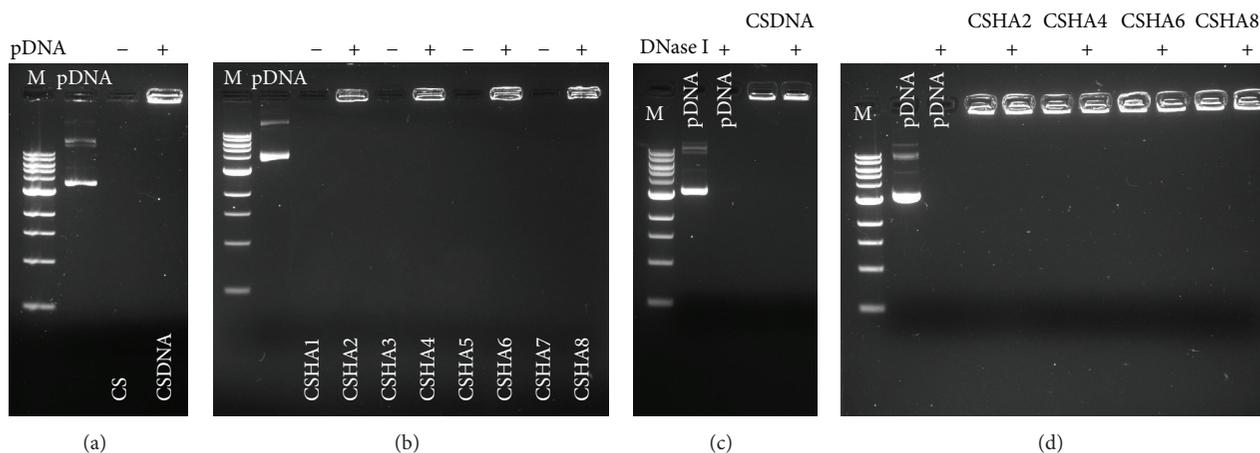


FIGURE 3: Polyplexes encapsulation efficiency and nuclease protection analyzed by 1% agarose gel electrophoresis, DNA visualized with ethidium bromide, (a) and (b) pDNA encapsulation in CSpDNA and CSHApDNA polyplexes, respectively, and lanes positive for polyplexes but negative for pDNA represent unloaded polyplexes. DNA is protected against DNase I digestion: (c) CSpDNA and (d) CSHApDNA polyplexes after incubation with DNase I.

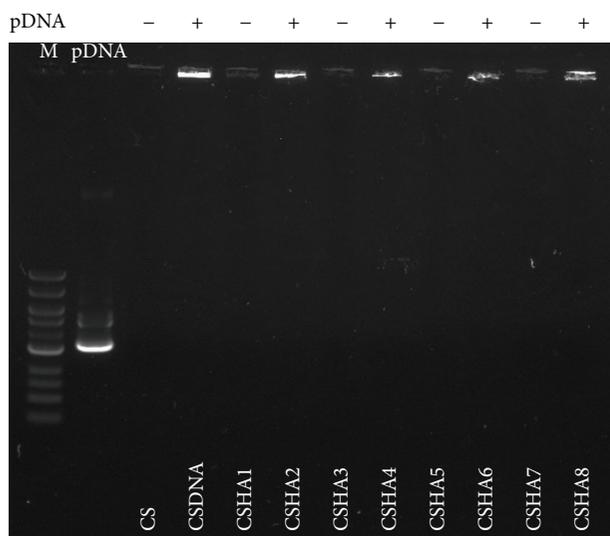


FIGURE 4: Polyplexes encapsulation efficiency analyzed by 0.3% agarose gel electrophoresis, DNA visualized with ethidium bromide, and lanes positive for polyplexes but negative for pDNA represent unloaded polyplexes.

there were no statistical differences in the size of CSDNA polyplexes, their PdI at 37°C started to increase, reflecting their decreasing stability at this temperature, after 49 days (Figure 6). Also, measurements of some formulations were discontinued due to visible aggregation and/or insufficient volume, hence the difference in the depicted time points.

As aimed, formulations containing HA and pDNA were less stable probably due to increased repulsion between charged groups. The stability of CSHA8 formulation decreased after 119 days at 4°C and much earlier at 37°C (63 days), as can be observed by the increase in size and PdI (Figure 6).

**3.4. In Vitro Studies.** Cytotoxicity of the developed polyplexes was evaluated using an MTT assay in the retinal cell line (ARPE-19, Figure 7). For HEK293 cells, our previous studies have indicated absence of cytotoxicity [9]. Statistical analysis revealed no differences between CS and CSHA formulations. Cell viability was above 75% regardless of formulation and concentration, except for the highest concentration tested, which caused a decrease in cellular viability, with values below 50% for all formulations. However, this concentration is above the range to be used *in vivo*.

One of the key points of our study was to evaluate if the introduction of HA would affect the stability of the polyplexes to facilitate DNA release and hence increase their transfection efficiency. CS and CSHA formulations with pDNA coding for GFP were tested both in the retinal cell line ARPE-19 and in HEK293 cells. The latter were used as a transfection control, as their permissibility in terms of transfection is widely accepted. As a control of the GFP expression system we used FuGene HD, a commercial reagent. The percentage of GFP positive cells 72 h after transfection is presented in Figure 8. Results showed that the transfection efficiency varied with cell line and type of formulation. HEK293 cells displayed higher levels of GFP expression when compared with the RPE cell line, as expected, based on their different mitotic rates. All formulations incorporating HA present increased GFP expression when compared to CS polyplexes, with CSHA polyplexes showing a near 4-fold increase in transfection efficiency in HEK293 cells.

## 4. Discussion

**4.1. Polyplexes.** This work aims to evaluate the effect that incorporating HA into CS polyplexes would have in the stability of the polyplexes and in their transfection efficiency. In summary, the characterization of the polyplexes revealed differences in size for pDNA loaded and unloaded polyplexes, but not between CS and CSHA polyplexes. This size difference

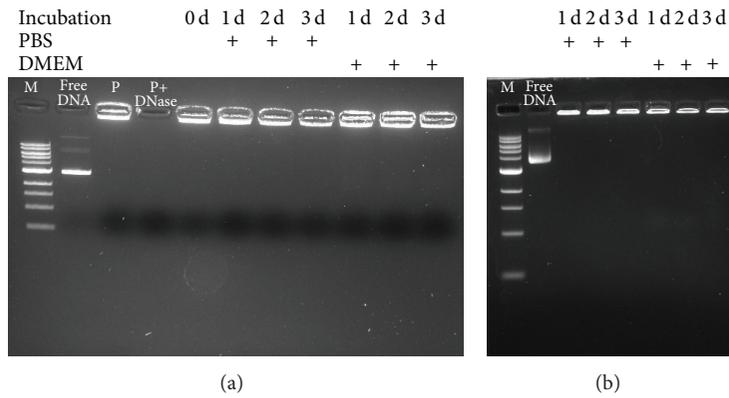


FIGURE 5: Polyplexes remain stable at physiological conditions, (a) CSpDNA and (b) CSHA pDNA polyplexes after incubation in PBS or DMEM with 10% FBS at 37°C. Polyplex pDNA retention analyzed by 1% agarose gel electrophoresis and pDNA visualized with ethidium bromide (P-polyplexes, P+DNase-polyplexes incubated with DNase I, data shown only for CSHA8 but representative of all CSHA polyplexes).

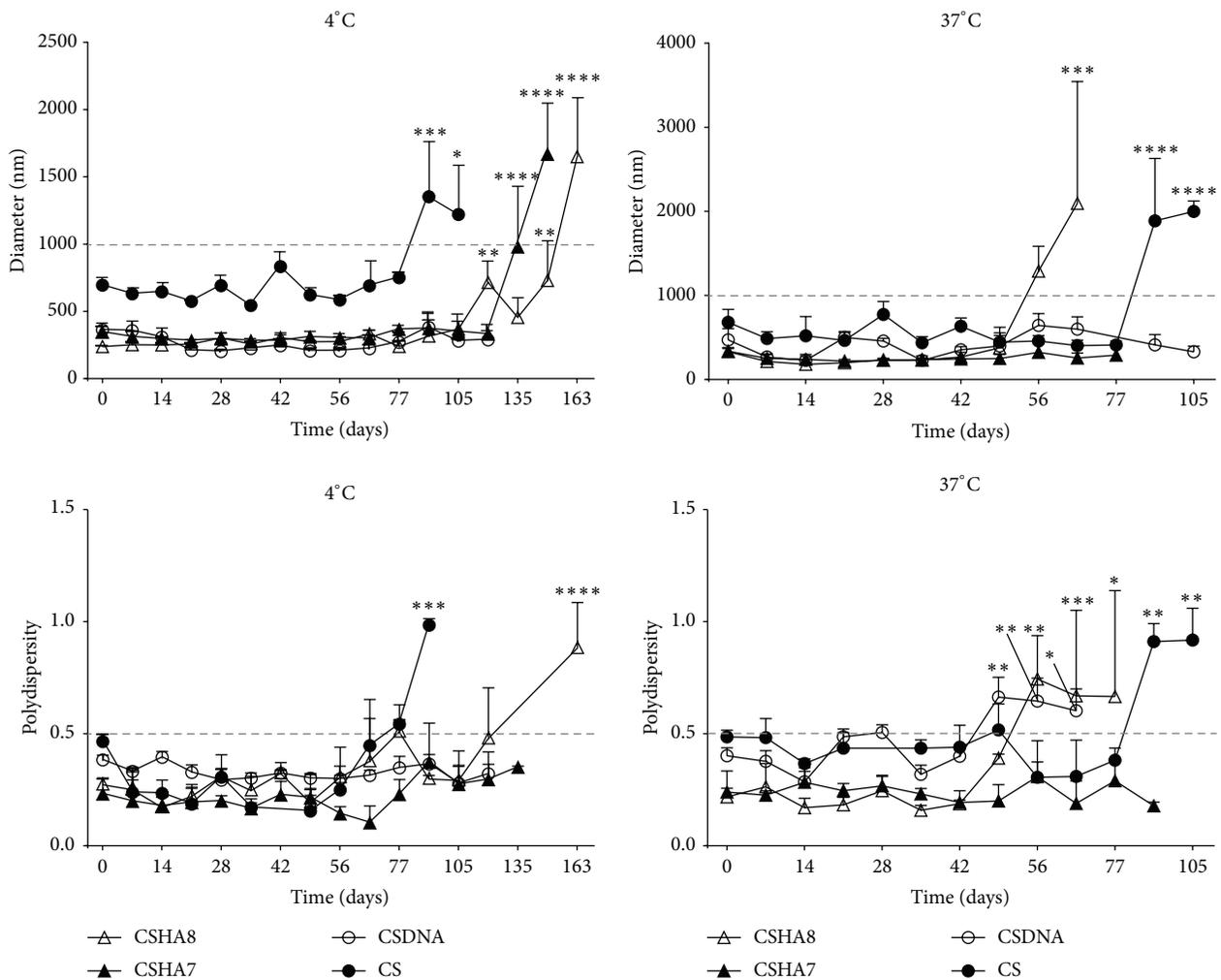


FIGURE 6: Particle stability at 4°C (left column) and 37°C (right column) as verified by continued monitoring of their size (upper row) and polydispersity (lower row). Significant differences to initial value were determined by Dunnett's multiple comparisons test (\*\*\*\*  $P < 0.0001$ , \*\*\*  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ ).

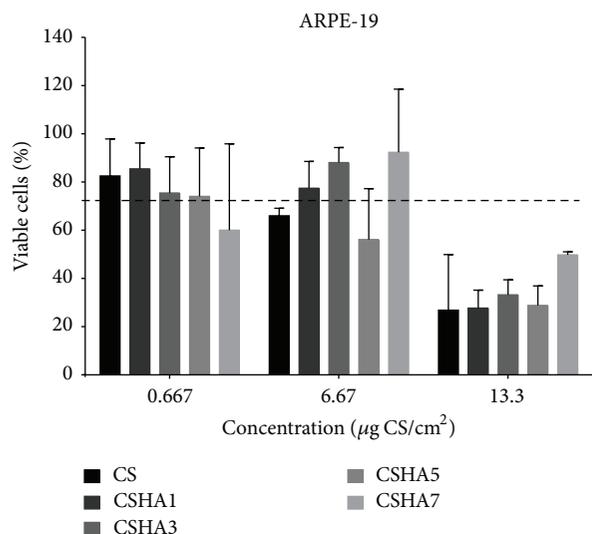


FIGURE 7: Cytotoxicity in RPE cell line (ARPE-19), given as percentage of viable cells after 72 h incubation with polyplexes at increasing concentrations. Polyplex concentration is given in total CS  $\mu\text{g}$  in polyplexes per  $\text{cm}^2$  of growth area.

between polyplexes with and without pDNA has been previously observed and is in accordance with the literature, since CS establishes strong interactions with negatively charged pDNA that contribute to a higher chain entanglement, thus producing smaller polyplexes [9].

Variations in size and surface charge were expected after the incorporation of HA into the formulation, since it has been shown previously that an increase in size and decrease in surface charge associated with HA content [12, 14]. In our study no effect of HA was observed in either size or surface charge. When addressing the effect of the MW of HA on the size of polyplexes, studies have shown the formation of smaller polyplexes with HA of increasing MW and also with the use of higher CS : HA ratios [11]. In this aspect, our results are well correlated with the literature, since using HA with a high MW produced polyplexes with smaller size and positive surface charge, whereas the use of low ratios increased sizes and decreased surface charge, causing aggregation (data not shown) as shown by Ducepe and Tabrizian (2009) [11].

It has also been suggested that the MW of the anionic polymer can influence the morphology of the polyplexes [11]. This was not observed in our study, since no morphological differences were noticeable either among CSHA formulations or between CS and CSHA polyplexes. We attribute this dissimilarity of our results with the literature to differences in the characteristics of the polymers and polyplexes such as MW and CS:HA ratios used in our study. Also, this difference in polyplex behavior might be related to differences in the localization and degree of incorporation of HA in the polyplexes [14]. During the chain entanglement process HA chains may have been trapped in the interior of the polyplex, thus not contributing to a significant decrease in surface charge.

**4.2. Stability.** Previous studies have shown that HA can influence the stability of polyplexes without affecting pDNA binding to CS [8]. Our results support these findings and also show that polyplexes remain stable at physiological conditions and are able to protect pDNA from nuclease degradation. Only in the long-term stability assay a decrease in polyplex stability was observed in CSHA polyplexes, more pronounced at 37°C.

**4.3. Cytotoxicity.** Polyplex charge is also an important parameter due to its relation to toxicity and transfection efficiency, as some authors have shown that a reduction in the positive charge results in an increase in transfection efficiency associated with a decrease in toxicity [18, 19]. A decrease in CS polyplex cytotoxicity with increasing amounts of HA in their formulations has been reported by De La Fuente et al. (2008), but our results show no such differences when either different CS:HA ratios or MW were used [14]. Since no decrease in surface charge was observed, we expected the incorporation of HA into the polyplexes to have no significant effect in the cytotoxicity. Nevertheless, considering the concentrations to be used *in vivo* our polyplexes can be considered a safe option for gene delivery.

**4.4. Transfection.** It is believed that one of the major causes of low transfection with polyplexes is their high stability and consequently inefficient and slow DNA delivery [8, 11]. Considering that all formulations displayed similar size, surface charge, and cytotoxicity, this suggests that the introduction of HA affected the interactions between CS and pDNA and modulated the release behavior, thereby resulting in a more efficient transfection. The observed transfection efficiency improvement over CS formulations may also be related to increased cellular internalization since it has been shown previously by de la Fuente that HA containing polyplexes can be internalized via interactions with the membrane CD44 receptor [14]. Recent studies with the same cell lines but using a lipid-based delivery system also containing HA corroborate our results and also support the idea that HA enhances the transfection efficiency by modulating the DNA condensation degree in the vector. The same study also states that the participation of the CD44 receptor in the internalization of the vectors is an important factor for increased transfection [20].

Similar to the results observed for HEK293 cells, a trend of higher GFP expression levels was observed when ARPE-19 were transfected with CSHA polyplexes, but it is not statistically significant. One possible explanation for this is their dividing rate, which is considerably lower than the one of HEK293 cells. The fact that cell lines with different mitotic rates were transfected demonstrates the versatility of our vectors and paves the way to possible future applications in other tissues. Additionally, it is known that the expression of CD44, the receptor for HA, in RPE is affected both by their proliferative state and by confluence, which might explain these results [21]. It has also been described that CD44 is increased in pathological conditions such as RPE wound repair and choroidal neovascularization [22, 23]. This would

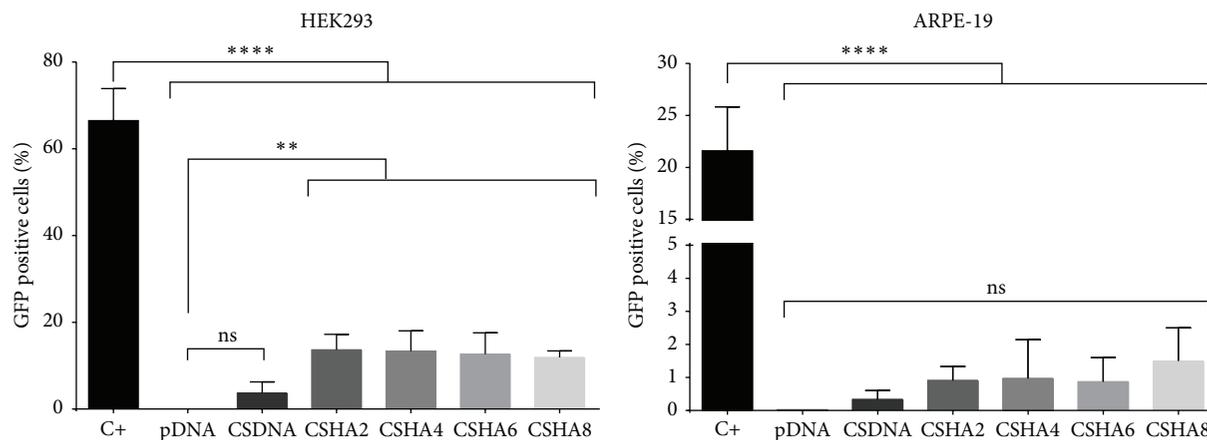


FIGURE 8: Transfection efficiency 72 h after transfection as percentage of GFP positive cells. Statistical differences were calculated using Tukey and Dunnett's multiple comparisons test (\*\*\*\*  $P < 0.0001$ , \*\*  $P < 0.01$ , ns—not significant).

suggest that polyplex CD44-dependent internalization *in vivo* and in a pathological situation could be greatly increased when compared with *in vitro* results. Based on this, our results lay the foundation for the use of CSHA polyplexes for gene delivery in animal models of retinal diseases.

## 5. Conclusions

In our study we describe the characterization of a hybrid polyplex for retinal nonviral gene therapy. The polyplexes containing both cationic (CS) and anionic (HA) polymers display an improved performance over the previously developed chitosan-based polyplexes. The incorporation of HA into the polyplexes resulted in a decrease in polyplex stability that most likely enabled a more rapid pDNA release whilst still protecting pDNA from degradation.

CSHA formulations showed an increase in *in vitro* cell transfection, which suggests that this strategy might be very effective *in vivo*, especially in retinal cells expressing CD44 and in pathological situations where CD44 is known to be involved, allowing cellular targeting by HA-containing formulations through CD44 interaction.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

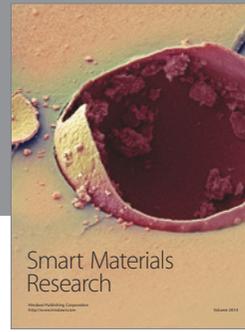
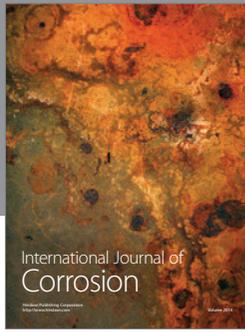
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