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Expression of MUC5AC in Ocular Surface Epithelial Cells Using Cationized Gelatin Nanoparticles

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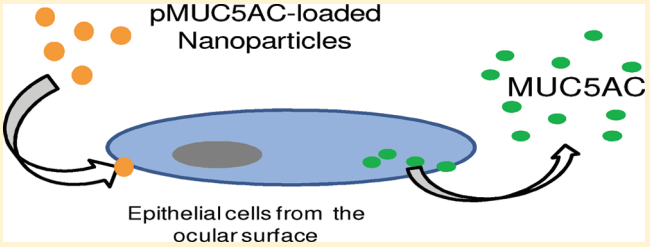
ABSTRACT: Decreased production of the mucin MUC5AC in the eye is related to several pathological conditions, including dry eye syndrome. A specific strategy for increasing the ocular levels of MUC5AC is not yet available. Using a plasmid specially designed to encode human MUC5AC, we evaluated the ability of hybrid cationized gelatin nanoparticles (NPs) containing polyanions (chondroitin sulfate or dextran sulfate) to transfect ocular epithelial cells. NPs were developed using the ionic gelation technique and characterized by a small size (<200 nm), positive zeta potential (+20/+30 mV), and high plasmid association efficiency (>95%). MUC5AC mRNA and protein were detected in conjunctival cells after in vitro transfection of the NPs. The in vivo administration of the NPs resulted in significantly higher MUC5AC expression in the conjunctiva compared to untreated control and naked plasmid. These results provide a proof-of-concept that these NPs are effective vehicles for gene therapy and candidates for restoring the MUC5AC concentration in the ocular surface.



KEYWORDS: nanoparticle, gene therapy, dry eye, ocular surface, MUC5AC

INTRODUCTION

The causes of dry eye are multifactorial and can be related to deficiencies in any of the components of the ocular surface and tear film. The tear film responds to very delicate regulatory mechanisms, notably those involving neuroregulatory phenomena and hormonal stimulation.[1] The inhibition of lacrimal gland secretion, decreased tear production, and hyperosmolar tears with the expression of several proinflammatory cytokines and chemokines are signs associated with dry eye syndrome. Among all of the factors related to tear film stability under normal conditions, a type of mucin called MUC5AC receives special attention because its decrease has been associated with several conditions, such as dry eye syndrome.[2,3]

Mucins are a class of heavily O-glycosylated glycoproteins in which the mass may reach 80% carbohydrate. As a result of their heavy glycosylation, mucins have been difficult to characterize, and it is only with the relatively recent application of molecular cloning techniques that mucin genes have been identified.[4] MUC5AC is a gel-forming mucin secreted by conjunctiva goblet cells. MUC5AC plays a key role in the homeostasis of lacrimal fluid, and alterations in either the distribution of the protein or its glycosylation have been described in dry eye syndrome.[5] Also, a reduction in the number of goblet cells that synthesize MUC5AC may correlate with decreased MUC5AC expression and constitute one possible mechanism responsible for tear instability.[6]

Despite the lack of options for increasing the level of MUC5AC when necessary, the in vivo delivery of exogenous gene that codifies the MUC5AC has potential for treating ocular diseases. Gene therapy has many advantages over conventional drugs because, once inside the cells, the genes are able to express their products for periods of time that greatly exceed the duration of action for currently available drugs.[7] The use of nanoparticles (NPs) as carriers of the therapeutic genetic materials for delivery to target tissues has become popular in recent years for the treatment of a wide range of ocular diseases. However, these systems are only effective with the right selection of biomaterials.[8,9]

We recently described the use of NPs produced by ionic gelation technique and based on gelatin cationized with spermine to efficiently transfect cornea cells. These NPs were able to protect the plasmid DNA from degradation. The properties of these NPs can be significantly improved by incorporating the polyanions chondroitin sulfate (CS) and dextran sulfate (DS). These systems have been shown to successfully induce plasmid internalization and are promising new carriers for gene delivery.[10]

The present study aimed to achieve successful transfection in the ocular surface using a new plasmid encoding a modified

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human MUC5AC protein associated with hybrid NPs based on cationized gelatin and CS or DS.

MATERIALS AND METHODS

Chemicals and Reagents. Type A gelatin (137 kDa) was purchased from Nitta Gelatin (Ontario, Canada). *N*-(3-Dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), tripolyphosphate (TPP), and spermine hydrochloride (SPM) were purchased from Sigma (Madrid, Spain). The pEGFP-c1 plasmid was obtained from Elim Biopharmaceutics (Hayward, CA). Plastic culture ware was obtained from Nunc (Roskilde, Denmark). DMEM/F12 culture medium and other cell culture reagents were from Invitrogen-Gibco (Inchinnan, U.K.). The plasmid pMUC5AC, codified for a modified MUC5AC protein and for the green fluorescent protein (GFP) as a marker, was designed by our group and supplied by Biomedal (Seville, Spain).[11]

Synthesis of Cationized Gelatin. The gelatin was cationized as described previously.[12] Briefly, a 1% (w/v) polymer solution in 0.1 M phosphate buffer (pH 5.3) was prepared, and for each mole of gelatin carboxyl groups, 3 mol of EDC and 50 mol of SPM were added. The final pH was adjusted to 5.0 and the solution reacted for 18 h in a warm bath at 37 °C. After the reaction, the final solution was dialyzed for 48 h, followed by lyophilization.

Preparation and Characterization of Nanoparticles. The NPs were formed using the ionic gelation technique as described previously.[13] Cationized gelatin (CG) was dissolved in water at a concentration of 1 mg/mL, CS at a concentration of 0.125 mg/mL, DS at 0.1 mg/mL, and TPP at varying concentrations (0.125 or 0.25 mg/mL). All solutions were sterilized by filtration (0.22 μm, MillexGV, Millipore, Billerica, MA). The plasmid and one of the anionic polymer solutions were incorporated with the TPP solution. NPs were obtained by adding the resulting mix to the CG solution with magnetic stirring at room temperature. The mean particle size was determined by photon correlation spectroscopy (PCS). The samples were diluted to the appropriate concentration. Each analysis was carried out at 25 °C with a detection angle of 173°. The zeta potential was obtained by laser Doppler anemometry (LDA), measuring the mean electrophoretic mobility. The samples were diluted with a millimolar solution of KCl. The PCS and LDA analyses were performed using a Zetasizer 3000HS (Malvern, Malvern, U.K.). The association efficiency was evaluated using the PicoGreen reagent (Quant-iT PicoGreen dsDNA Assay Kit, Molecular Probes, Madrid, Spain) according to the manufacturer's directions. The amount of free pDNA was quantified using the nanoparticle supernatant obtained after centrifugation in a Microfuge 22-R (Beckmann Coulter, Krefeld, Germany) for 30 min at 12,000 rpm. For the in vivo studies, the nanoparticles were optionally concentrated by centrifugation (Beckman CR412, Beckman Coulter) at 10,000 rcf for 30 min at 4 °C with 0.1% glycerol (v/v). The NPs were then resuspended in 5% glucose at a final concentration of 0.5 μg/μL of plasmid.

Cell Culture. Two different cell lines were used. The IOBA-NHC cell line[14] is a nontransfected, spontaneously immortalized conjunctival epithelial cell line used in passages 71 to 87. Cells were grown in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 5000 U/mL penicillin, 5 mg/mL streptomycin, 2.5 μg/mL fungizone, 2 ng/mL human epidermal growth factor (EGF), 1 μg/mL bovine insulin, 0.1 μg/mL cholera toxin, and 0.5 μg/mL hydrocortisone. The HCE cell line[15] is a SV40-immortalized human corneal epithelial cell line kindly gifted by Professor Arto Urti (University of Helsinki, Finland). Cells from passages 42 to 52 were cultured in DMEM/F-12 supplemented with 15% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 ng/mL EGF, 0.5% DMSO, 5 μg/mL insulin, and 0.1 μg/mL cholera toxin. Both cell lines were cultured at 37 °C in a 5% CO_2—95% air atmosphere. Media were changed every other day, and daily observations were made by phase contrast microscopy.

Transfection Studies. In order to evaluate the ability of the pMUC5AC-loaded NPs to transfect HCE and IOBA-NHC cell lines, cells were seeded onto 24-well plates (8×10^5 cells/well) and grown for 24 h reaching a final confluence of 75%. Then HCE and IOBA-NHC cells were incubated with the NPs for 3 h (5 μg of pDNA/well). Controls included cells alone and cells transfected with the naked pDNA (1 μg of pDNA/well) using JetPEI—RGD (Polyplus Transfections, Illkirch, France) as transfection reagent. The expression of GFP was evaluated 72 h post-transfection using an inverted fluorescence microscope (Leica DMI 6000B, Wetzlar, Germany). Observations were made and images captured using a 40× objective.

Cell Viability. The viability of NP-exposed cells was measured using the XTT toxicity test (Sigma).[16]Cells were seeded onto 96-well plates (2×10^5 cells/well) and grown until 75% confluence. HCE and IOBA-NHC cells were incubated with the NPs for 3 h (5 μg of pDNA/well). Culture medium was replaced with fresh phenol red-free RPMI, 72 h after NP incubation. Then XTT solution was added, and cells were incubated at 37 °C for 15 h. Plates were read in a SpectraMAXM5 multidetection microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm (reference wavelength: 620 nm). Controls included cells alone and cells exposed to 0.5% benzalkonium chloride (BKC), which induces a significant decrease of ocular cell viability in concentrations greater that 0.05%.[17] Cell viability was calculated as a percentage with regard to control cells. Each test was repeated three times in quadruplicate.

Real Time RT-PCR of MUC5AC. MUC5AC mRNA expression was quantified using real time RT-PCR. Total RNA from NP-exposed cells and control cells were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. The RNA concentration was measured using the Quant-it RNA Assay Kit (Invitrogen, Barcelona, Spain), and it was stored at −80 °C before use. cDNA was generated from 1 μg of total RNA with the SuperScript VilocDNA kit (Invitrogen), according to the manufacturer's protocol.

Real time RT-PCR was performed on the 7500 Real Time System (Applied Biosystem, Foster City, CA) using SYBR Green (Applied Biosystem) to quantify levels of MUC5AC mRNA in the different treatment groups. For this purpose MUC5AC primers (forward 5′-CCCACAGAACCCAGTACAA-3′ and reverse 5′-AATGTGTAGCCCTCGTCT-3′) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward 5′-GAAGGTGAAGGTCGGAGTCAAC-3′ and reverse 5′-CTGGAAGATGGTGATGGGATTTC-3′) were used. Relative quantification of the signals was done by normalizing the signal of MUC5AC mRNA with the GAPDH signal. The expression was referred to that in control cells.

ELISA of MUC5AC. MUC5AC protein was quantified by enzyme-linked immunosorbent assay (ELISA).[18] Cell lysates (50 μL) were incubated with bicarbonate—carbonate buffer (50 μL) (0.05 M, pH 9.6) at 37 °C overnight in MaxiSorp flat-bottom 96-well plates (Nunc) until dry. Plates were washed three times with PBS and blocked with 2% bovine serum albumin (BSA fraction V; Sigma, Madrid, Spain) for 1 h at room temperature. Plates were again washed three times with PBS

and then incubated with 50 μL of mouse anti-MUC5AC antibody (Chemicon, Billerica, MA), which was previously diluted (1:500) in PBS with 0.05% Tween 20. After 1 h, the wells were washed three times with PBS and 100 μL of horseradish peroxidase-conjugated secondary donkey anti-mouse IgG antibody (Jackon Inmuno Res., Madrid, Spain) previously diluted (1:10,000) in PBS with 0.05% Tween 20 and 0.1% BSA, added to each well. After 1 h, the plate was washed three times with PBS. Color reaction was developed with 3,3′,5,5′-tetramethylbenzidine peroxide (TMB) solution (Invitrogen) and stopped with 1 M H_2SO_4. Plates were read in a SpectraMAXM5 multidetection microplate reader (Molecular Devices, Sunnyvale, CA) at 450 nm. MUC5AC analogue B (AnaSpec, Fremont, CA) was used as a standard. The obtained values were normalized to the total amount of protein quantified by the BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturer's directions. The results were expressed as a percent of the expression of MUC5AC in the untreated control. Similar determination was performed in cell culture media (500 μL).

In Vivo Assay. In vivo studies adhered to the Association for Research in Vision and Ophthalmology statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the institutional research committee. To evaluate the ability of pMUC5AC-loaded NPs to produce an efficient transfection in vivo, normal conscious New Zealand rabbits were placed in a restraint box and 30 μL of concentrated NP dispersion (0.5 μg/μL of pDNA) was administrated topically into the cul-de-sac. The dispersion was applied every 30 min until reaching 75 μg of plasmid. To facilitate the administration, the NPs were previously 10-fold concentrated. Naked pMUC5AC was used as control, following the same protocol, and all results were compared to control animals treated with 5% glucose solution. Also, the in vivo tolerance to pMUC5AC-loaded NPs ocular exposure was studied. Clinical signs (ocular discomfort, presence of corneal and/or conjunctival alterations, discharge and eyelid swelling) were macroscopically evaluated 4 days post-transfection. Animals were euthanized 4 days post-transfection and corneal and conjunctival tissues collected. Tissues were chopped by scissors and were homogenized. Expression of MUC5AC in cornea and conjunctiva was evaluated by ELISA.

Statistical Analysis. The results were expressed as mean ± standard deviation (SD) of three independent experiments and statistically analyzed by ANOVA, followed by the Tukey test.

RESULTS

Preparation and Characterization of the Nanoparticles. Hybrid NPs were recently developed as a nonviral delivery system for ocular genes. We evaluated the ability of such hybrid systems in the transfection of a plasmid encoding MUC5AC. All of the systems were based on cationized gelatin and reticulated with TPP: (i) CG NPs, (ii) CG/CS NPs, and (iii) CG/DS NPs. All systems were less than 150 nm in size, and positive zeta potential was between +20 and +30 mV (Table 1). The amount of pDNA associated with the nanoparticles determined using the PicoGreen reagent showed a high association efficiency of over 90% for the systems.

In Vitro Transfection Studies. To evaluate the ability of pMUC5AC-loaded NPs to produce an efficient transfection of HCE and IOBA-NHC cells, first GFP expression was evaluated by fluorescence microscopy and then MUC5AC mRNA and protein expressions were measured by real time RT-PCR and ELISA, respectively. In addition, cell viability was measured in both cell lines 72 h after NP incubation. There were no significant differences in cell viability between control HCE and IOBA-NHC cells and those exposed to the three formulations (Figure 1). Both HCE and IOBA-NHC cells incubated with NPs had a well-preserved morphology that was similar to controls, as determined by phase contrast microscopy. Even without statistical difference from the control, a tendency to decrease the cell viability can be observed for JetPEI−RGD complexes.

GFP expression evaluated 72 h post-transfection showed a little fluorescence in cells transfected with the positive transfection control JetPEI−RGD, but no fluorescence was detected in the HCE and IOBA-NHC cells after transfection with the NPs (data not shown).

MUC5AC mRNA Expression. Both HCE and IOBA-NHC cell lines showed detectable MUC5AC mRNA expression in cells exposed to pMUC5AC-JetPEI−RGD complexes and to the 3 formulations, as determined by real time RT-PCR. This expression was significantly higher in pMUC5AC-JetPEI−RGD exposed cells than in cells treated with the formulations. The 3 formulations, CG, CG/CS, and CG/DS, produced an efficient transfection of MUC5AC in both cell lines, influenced by the presence of the anionic polymer in the formulation (Figure 2). MUC5AC mRNA expression in HCE cells following incubation with CG NPs (1091 ± 232) was 2-fold higher than the expression induced by CG/CS (543 ± 240) or CG/DS (407 ± 152) in this cell line. In IOBA-NHC cells, the presence of CS in the formulation significantly increased the expression of MUC5AC (198 ± 36), which was 2- and 3-fold greater than the expression induced by CG (106 ± 17) and CG/DS (69 ± 19) NPs, respectively.

MUC5AC Protein Expression. MUC5AC protein expression in cell lysates was achieved in both cell lines when using the commercial transfection agent JetPEI−RGD, as determined by

Table 1. Characteristics of 7.5% pMUC5AC-Loaded Nanoparticles[a]

formulation	mass ratio	size (nm)	ξ (mV)	AE (%)
CG/TPP	12:1	131 ± 3	+22 ± 2	99.2 ± 0.5
CG/CS/TPP	12:1:0.5	135 ± 14	+33 ± 3	97.6 ± 1.3
CG/DS/TPP	15:1:0.6	106 ± 4	+31 ± 2	95.7 ± 3.2

[a] CG, cationized gelatin with spermine; AE, association efficiency; CS, chondroitin sulfate; DS, dextran sulfate; TPP, tripolyphosphate. Data are mean ± SD; n = 3.

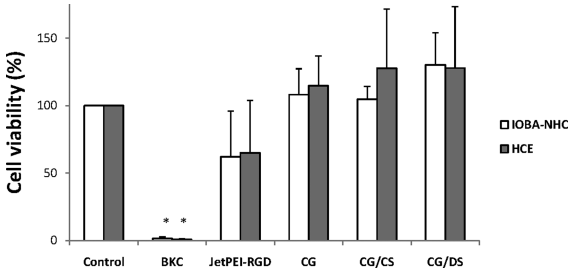

Figure 1. Cell viability 72 h after NPs treatment in HCE and IOBA-NHC cells. Results are expressed as % of control. Benzalkonium chloride (BKC) was use as toxicity control. There were no significant differences between NP-treated and nontreated corneal and conjunctival cells. JetPEI−RGD was used as commercial transfection agent control. n = 3 independent experiments in triplicate, data are mean ± SD. *p < 0.05 vs control.

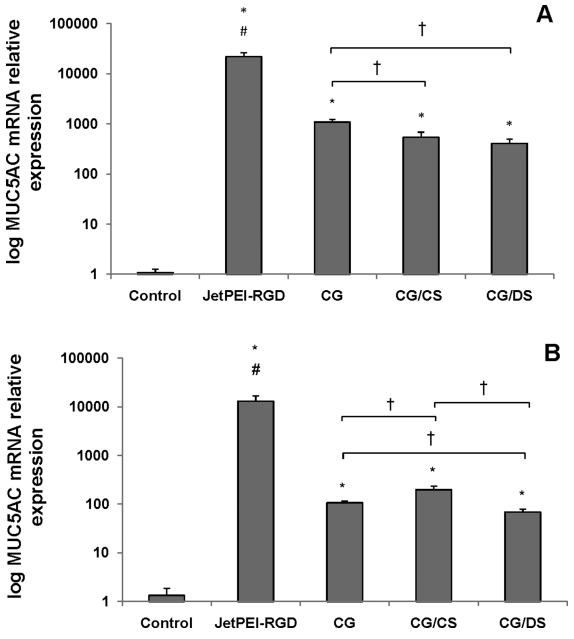

Figure 2. Relative expression of MUC5AC mRNA was quantified by real time RT-PCR in HCE cells (A) and in IOBA-NHC cells (B) 72 h post-transfection. Untreated cells (Control) and pMUC5AC-JetPEI−RGD (JetPEI-RGD) were used as a control. CG NPs (CG), CG/CS NPs (CG/CS), and CG/DS NPs (CG/DS) were used to transfect the cells. $n = 3$ independent experiments in triplicate, data are mean ± SD. $^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs all formulations, $^{\dagger}p < 0.05$ between formulations.

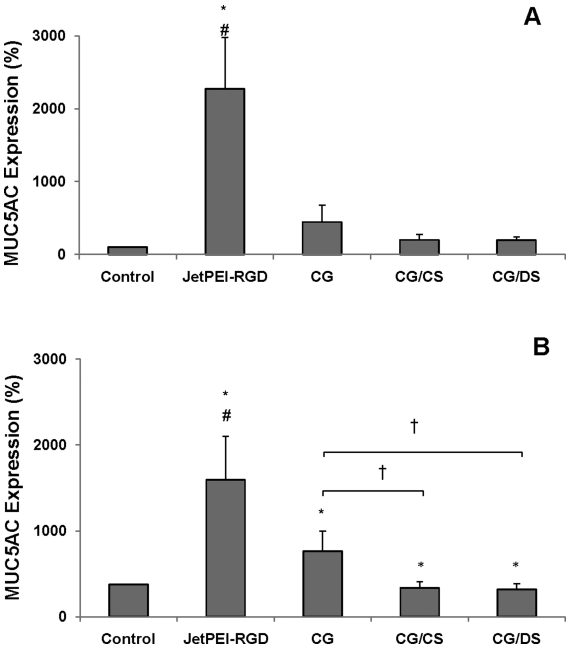

Figure 3. Relative expression of MUC5AC was quantified by ELISA in HCE cells (A) and in IOBA-NHC cells (B) 72 h post transfection. Untreated cells (Control) and pMUC5AC-JetPEI−RGD (JetPEI-RGD) were used as a control. CG NPs (CG), CG/CS NPs (CG/CS), and CG/DS NPs (CG/DS) were used to transfect the cells. $n = 3$ independent experiments in triplicate, data are mean ± SD. $^{*}p < 0.05$ vs control, $^{\#}p < 0.05$ vs all formulations, $^{\dagger}p < 0.05$ between formulations.

ELISA. This expression was always significantly higher than that of untreated cells and cells exposed to the formulations. In IOBA-NHC cells, the level of expression was higher ($p < 0.05$) for CG NPs (764 ± 321) compared to the NPs possessing polyanions: CG/CS (337 ± 120) and CG/DS (317 ± 137) (Figure 3). Although mRNA production was induced in HCE cells, none of the formulations were capable of inducing protein expression. Additional data indicate secretion of the MUC5AC in the culture media by HCE and IOBA-NHC cells after transfection. For the IOBA-NHC cells the JetPEI−RGD resulted in higher secretion of MUC5AC (14.4 ± 0.3 μg/mL) when compared to the systems. In the case of the formulations, higher secretion was achieved by CG/CS NPs (12.5 ± 1.0 μg/mL) and CG NPs (10.5 ± 0.3 μg/mL) followed by CG/DS NPs (4.2 ± 5.2 μg/mL). No MUC5AC was detected in the culture media of HCE cells after NP incubation, while some levels were detected for the JetPEI−RGD complexes (10.6 ± 1.1 μg/mL).

In Vivo Transfection Study. An in vivo assay was performed to evaluate the effect of pMUC5AC-loaded NP transfection on the expression of MUC5AC on the eye surface tissues. CG NPs, the system with the best response in terms of protein expression in conjunctiva cells, were applied onto the eyes of New Zealand rabbits.

The macroscopic evaluation showed that pMUC5AC-loaded NPs are well tolerated. No animal showed ocular discomfort, irritation or swelling. Only a mild nasal discharge and increased blinking immediately after instillations were observed in few rabbits. In addition, there were no macroscopic alterations of the ocular surface structures after pMUC5AC-loaded NP exposure. No edema, redness, or corneal vascularization was observed in treated eyes.

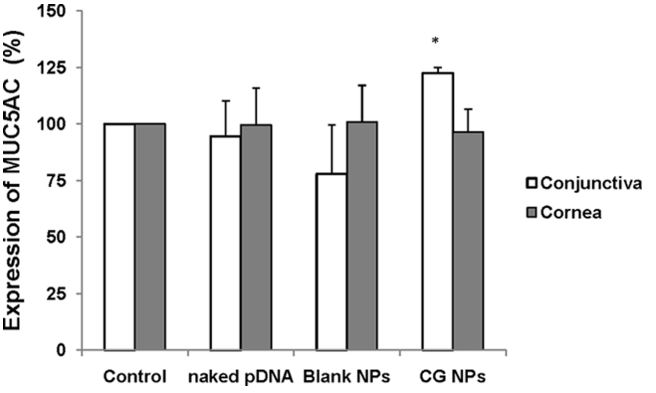

Figure 4. Expression of MUC5AC in rabbit cornea (white bars) and conjunctiva (gray bars) 4 days after transfection with cationized gelatin loaded with pMUC5AC (CG NPs), blank nanoparticles (blank NPs) or naked pDNA. $n = 3$ independent experiments in triplicate, data are mean ± SD. $^{*}p < 0.05$ vs control.

Neither the naked plasmid nor the blank NPs induced MUC5AC expression in any tissue (Figure 4). Meanwhile, the treatment with the cationized gelatin NPs resulted in significant MUC5AC expression in the conjunctiva (122 ± 4%; $p < 0.05$), but not in the cornea. In the in vitro tests, only the conjunctiva was able to express MUC5AC after transfection, too.

DISCUSSION

Although these NPs were described previously, they were associated with a model plasmid (pEGFP) very different from pMUC5AC. Despite the huge difference in the plasmid size

(13.1 Kb for pMUC5AC vs 5.3 Kb for pEGFP), no difference in the NP size was observed, probably due to the capacity of spermine to condense the plasmid.[19] The high association efficiency can also be attributed to the high interaction between DNA and the spermine linked to the gelatin backbone.[20−22] The positive zeta obtained for all the systems is important for increasing the stability of the system[23] and improving the interaction between NPs and the eye surface, increasing the transfection efficiency.[24] The viability of treated HCE and IOBA-NHC cells was totally preserved (Figure 1). This is in agreement with previous in vitro works for these nanosystems,[10] indicating an adequate safety level for their application in the ocular surface.

Even though the overexpression of MUC5AC has been reported in other organs and tissues, such as the prostate and airways, it is generally associated with pathological conditions.[25,26] However, the pMUC5AC-loaded NPs reported here are the first therapy aimed at specifically increasing MUC5AC levels in the eye. This increase in MUC5AC concentrations would be of great importance to improve the lacrimal fluid quality in dry eye therapy. Among the different ways to evaluate the MUC5AC expression carried out, only the indirect qualitative evaluation of GFP expression has failed. Some previous studies have used GFP expression constructs in their systems and also failed to detect fluorescence. This failure could be due to numerous reasons, including GFP expression below the limit of detection and the failure in forming the chromophore.[27] Meanwhile, increases in the mRNA and protein levels were detected in the IOBA-NHC and HCE cell lines. It is important to emphasize that reduced MUC5AC mRNA expression has been related to dry eye conditions, such as Sjögren's syndrome.[6] The increase of the protein expression detected in the IOBA-NHC represents a significant advance since, thus far, the only way to induce the overexpression of MUC5AC was by using proinflammatory cytokines and other stimulating factors that cannot be used as therapeutic agents for their several biological undesired responses.[25,26] Notably, the mRNA and protein expression data are pictures of two different and sequential events analyzed at the same time (day 3 post-transfection). One explanation for the difference detected between mRNA and protein production in IOBA-NHC cells is that CG/CS NPs induce longer and lasting expression. So, the possible reason why the mRNA levels at day 3 are higher but CG NPs have a burst effect on expression is that a higher level of MUC5AC is expressed, though just for a few days. Expression of the protein was achieved in HCE cells when using the commercial transfection agent, but not with any of the formulations; thus, possible reasons for the lack of expression in this cell line may be related to the fact that the level of synthesized mRNA was not enough to produce detectable amounts of protein in 72 h.[28,29] On the other hand, studies performed in the cell culture media also revealed the presence of MUC5AC. Taking into account the fact that MUC5AC is not normally expressed in these epithelial cells (it is just expressed by the goblet cells), these results evidenced that using the developed plasmid-loaded nanosystems the protein is not only produced by the cultured epithelial cells but also secreted by these cells to the cell culture media.

Even after successful in vitro transfection the NPs have been concentrated since higher amounts of pDNAs are usually required for in vivo expression.[30] The concentration by using centrifugation is a well-described strategy and resulted in no changes in the physicochemical properties of the system.[31] Nasal discharge was probably because of certain drugs' capability of traveling to nasal mucosa after being drained from the ocular surface of treated eyes. Increased blinking could be related to the known irritative effect caused by the instillation, a response that is also observed after a simple buffer solution instillation.[32] The inefficiency of the naked plasmid in transfected conjunctiva or cornea highlights the need for an efficient vehicle to deliver the plasmid (Figure 4). Within this frame, it has been described that some nanoparticulate matter is capable of stimulating the expression of MUC5AC in lungs.[33] However, this probably occurs as a protective response since inflammation was also observed. In fact, as also evidenced (Figure 4), the developed blank NPs did not induce MUC5AC expression. Therefore, the statistically significant ($p < 0.05$) protein levels elicited by the nanoparticles associated with the pMUC5AC plasmid can be exclusively attributed to the correct expression of the plasmid. The expression of the protein MUC5AC only took place in conjunctiva, the result being similar to that obtained in vitro for IOBA-NHC cells. The lack of expression of MUC5AC in the cornea is desirable, conjunctiva being the natural target for MUC5AC expression (goblet cells of the conjunctiva). Indeed, no natural expression of MUC5AC has been reported in corneal structures, thus far.

CONCLUSION

The elevation of MUC5AC levels was achieved for the first time using a new plasmid and cationized gelatin nanoparticles as vehicles. We showed that the nanocarriers successfully transfected rabbit conjunctiva in vivo and were essential for the expression of the mucin from the associated plasmid. Therefore, these gelatin nanoparticles associated with the pMUC5AC plasmid are promising new medicines for restoring MUC5AC levels, creating new possibilities for treating several pathological conditions in which the restoration of these levels is beneficial, such as dry eye.

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REFERENCES

(1) Baudouin, C. The Pathology of Dry Eye. *Surv Ophthalmol.* **2001**, *45* (Suppl. 2), S211–S220.

(2) Calonge, M. C.; Corrales, R. M.; Sáez, V.; Mayo, A.; Herreras, J. M.; Stern, M. E.; Chaves, F. J. Alteration of conjunctival mucin gene

expression in tear-deficient dry eye syndrome. *Invest. Ophthalmol. Visual Sci.* **2003**, *44*, 2508–B347.

(3) Mantelli, F.; Argüeso, P. Functions of ocular surface mucins in health and disease. *Curr. Opin. Allergy Clin. Immunol.* **2008**, *8* (5), 477–483.

(4) Gipson, I. K. The Ocular Surface: The Challenge to Enable and Protect Vision. *Invest. Ophthalmol. Visual Sci.* **2007**, *48* (10), 4391–4398.

(5) Danjo, Y.; Watanabe, H.; Tisdale, A. S.; George, M.; Tsumura, T.; Abelson, M. B.; Gipson, I. K. Alteration of Mucin in Human Conjunctival Epithelia in Dry Eye. *Invest. Ophthalmol. Visual Sci.* **1998**, *39* (13), 6202–6209.

(6) Argüeso, P.; Balaram, M.; Spurr-Michaud, S.; Keutmann, H. T.; Dana, M. R.; Gipson, I. K. Decreased Levels of the Goblet Cell Mucin MUC5AC in Tears of Patients with Sjögren Syndrome. *Invest. Ophthalmol. Visual Sci.* **2002**, *43* (4), 1004–1011.

(7) Borrás, T. Recent developments in ocular gene therapy. *Exp. Eye Res.* **2003**, *76*, 643–652.

(8) Cai, X.; Conley, S.; Naash, M. Nanoparticle applications in ocular gene therapy. *Vision Res.* **2007**, *48* (3), 319–324.

(9) Coelho, J. F.; Ferreira, P. C.; Alves, P.; Cordeiro, R.; Fonseca, A. C.; Góis, J. R.; Gil, M. H. Drug delivery systems: Advanced technologies potentially applicable in personalized treatments. *EPMA J.* **2010**, *1*, 164–209.

(10) Zorzi, G. K.; Párraga, J. E.; Seijo, B.; Sánchez, A. Design of hybrid nanoparticles based on cationized gelatin and the polyanions dextran sulfate and chondroitin sulfate for ocular gene therapy. *Macromolecular Bioscience* **2011**, DOI: 10.1002/mabi.201100005.

(11) Párraga, J. E.; Zorzi, G. K.; Paolicelli, P.; Seijo, B.; Sánchez, A.; Contreras, L.; Diebold, Y. Nanoparticulas para la prevención y/o el tratamiento de enfermedades de las mucosas. Spanish Pat., P201031678, 2010.

(12) Kushibiki, T.; Tomoshige, R.; Fukunaka, Y.; Kakemi, M.; Tabata, Y. In vivo release and gene expression of plasmid DNA by hydrogels of gelatin with different cationization extents. *J. Controlled Release* **2003**, *90*, 207–216.

(13) Párraga, J. E.; Zorzi, G. K.; Seijo, B.; Sánchez, A. Gelatin nanoparticles for ocular gene delivery. *Hum. Gene Ther.* **2009**, *20* (11), 1545.

(14) Diebold, Y.; Calonge, M.; Enríquez de Salamanca, A.; Callejo, S.; Corrales, R. M.; Sáez, V.; Siemasko, K. F.; Stern, M. E. Characterization of a Spontaneously Immortalized Cell Line (IOBA-NHC) from Normal Human Conjunctiva. *Invest. Ophthalmol. Visual Sci.* **2003**, *44*, 4263–4271.

(15) Araki-Sasaki, K.; Ohashi, Y.; Sasabe, T.; Hayashi, K.; Watanabe, H.; Tano, Y.; Handa, H. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest. Ophthalmol. Visual Sci.* **1995**, *36* (3), 614–621.

(16) Roehm, N. W.; Rodgers, G. H.; Hatfield, S. M.; Glasebrook, A. L. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J. Immunol. Methods* **1991**, *142*, 257–267.

(17) Pauly, A.; Meloni, M.; Brignole-Baudouin, F.; Warnet, J. M.; Baudouin, C. Multiple endpoint analysis of the 3D-reconstituted corneal epithelium after treatment with benzalkonium chloride: early detection of toxic damage. *Invest. Ophthalmol. Visual Sci.* **2009**, *50*, 1644–1652.

(18) Takeyama, K.; Dabbagh, K.; Lee, H. M.; Agustí, C.; Lausier, J. A.; Ueki, I. F.; Grattan, K. M.; Nadel, J. A. Epidermal growth factor system regulates mucin production in airways. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96* (6), 3081–3086.

(19) Wilson, R. W.; Bloomfield, V. A. Counterion-Induced Condensation of Deoxyribonucleic Acid. A Light-Scattering Study. *Biochemistry* **1979**, *18*, 2192–2196.

(20) D'Agostino, L.; Di Pietro, M.; Di Luccia, A. Nuclear aggregates of polyamines are supramolecular structures that play a crucial role in genomic DNA protection and conformation. *FEBS J.* **2005**, *272*, 3777–3787.

(21) Feurstein, B. G.; Pattabiraman, N.; Marton, L. J. Spermine-DNA interactions: a theoretical study. *Proc. Natl. Acad. Sci. U.S.A.* **1986**, *83*, 5948–5952.

(22) Vijayanathan, V.; Thomas, T.; Antony, T.; Shirahata, A.; Thomas, T. J. Formation of DNA nanoparticles in the presence of novel polyamine analogues: a laser light scattering and atomic force microscopic study. *Nucleic Acids Res.* **2004**, *32*, 127–134.

(23) Mora-Huertas, C. E.; Fessi, H.; Elaissari, A. Polymer-based nanocapsules for drug delivery. *Int. J. Pharm.* **2010**, *385* (1−2), 113–42.

(24) Rabinovich-Guilatt, L.; Couvreur, P.; Lambert, G.; Dubernet, C. Cationic Vectors in Ocular Drug Delivery. *J. Drug Targeting* **2004**, *12* (9−10), 623–633.

(25) Fujisawa, T.; Velichko, S.; Thai, P.; Hung, L. Y.; Huang, F.; Wu, R. Regulation of airway MUC5AC expression by IL-1beta and IL-17A; the NF-kappaB paradigm. *J. Immunol.* **2009**, *183* (10), 6236–6243.

(26) Zhao, J.; Maskrey, B.; Balzar, S.; Chibana, K.; Mustovich, A.; Hu, H.; Trudeau, J. B.; O'Donnell, V.; Wenzel, S. E. Interleukin-13-induced MUC5AC is regulated by 15-lipoxygenase 1 pathway in human bronchial epithelial cells. *Am. J. Respir. Crit. Care Med.* **2009**, *179* (9), 782–790.

(27) Kain, S. R.; Kitts, P. Expression and Detection of Green Fluorescent Protein (GFP). *Methods Mol. Biol.* **1997**, *63* (Part III), 305–324.

(28) Chen, G.; Gharib, T. G.; Huang, C.; Taylor, J. M. G.; Misek, D. E.; Kardia, S. L. R.; Giordano, T. J.; Iannettoni, M. D.; Orringer, M. B.; Hanash, S. M.; Beer, D. G. Discordant Protein and mRNA Expression in Lung Adenocarcinomas. *Mol. Cell Proteomics* **2002**, *12*, 304–313.

(29) Gry, M.; Rimini, R.; Strömberg, S.; Asplund, A.; Pontén, F.; Uhlén, M.; Nilsson, P. Correlations between RNA and protein expression profiles in 23 human cell lines. *BMC Genomics* **2009**, *10* (365). DOI: 10.1186/1471-2164-10-365.

(30) De la Fuente, M.; Seijo, B.; Alonso, M. J. Bioadhesive hyaluronan-chitosan nanoparticles can transport genes across the ocular mucosa and transfect ocular tissue. *Gene Ther.* **2008**, *15* (9), 668–676.

(31) De la Fuente, M.; Seijo, B.; Alonso, M. J. Novel hyaluronan-based nanocarriers for transmucosal delivery of macromolecules. *Macromol. Biosci.* **2008**, *8* (5), 441–450.

(32) Sussman, J. D.; Friedman, M. P. Irritation of rabbit eye caused by contact lens wetting solutions. *Am. J. Ophthalmol.* **1969**, *68*, 703–706.

(33) Inoue, K.; Takano, H.; Yanagisawa, R.; Sakurai, M.; Abe, S.; Yoshino, S.; Yamaki, K.; Yoshikawa, T. Effects of nanoparticles on lung physiology in the presence or absence of antigen. *Int. J. Immunopathol. Pharmacol.* **2007**, *20* (4), 737–44.