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Novel water-soluble mucoadhesive carbosilane dendrimers for ocular administration

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Abstract

Purpose: To determine the potential use of water-soluble anionic and cationic carbosilane dendrimers (Generations 1-3) as mucoadhesive polymers in eyedrop formulations. **Methods:** Cationic carbosilane dendrimers decorated with ammonium $-\text{NH}_3^+$ groups were prepared by hydrosilylation of Boc-protected allylamine and followed by deprotection with HCl. Anionic carbosilane dendrimers with terminal carboxylate groups were also employed in this study. *In vitro* and *in vivo* tolerance studies were performed in human ocular epithelial cell lines and rabbit eyes respectively. The interaction of dendrimers with transmembrane ocular mucins was evaluated with a surface biosensor. As proof of concept, the hypotensive effect of a carbosilane dendrimer eyedrop formulation containing acetazolamide (ACZ), a poorly water-soluble drug with limited ocular penetration, was tested after instillation in normotensive rabbits. **Results:** The methodology used to synthesize cationic dendrimers avoids the difficulty of obtaining neutral $-\text{NH}_2$ dendrimers that require harsher reaction conditions and also present high aggregation tendency. Tolerance studies demonstrated that both prototypes of water-soluble anionic and cationic carbosilane dendrimers were well tolerated in a range of concentrations between 5 and 10 μM . Permanent interactions between cationic carbosilane dendrimers and ocular mucins were observed using biosensor assays, predominantly for the generation-three (G3) dendrimer. An eyedrop formulation containing G3 cationic carbosilane dendrimers (5 μM) and ACZ (0.07%) (289.4 mOsm; 5.6 pH; 41.7 mN/m) induced a rapid (onset time 1 h) and extended (up to 7 h) hypotensive effect, and led to a significant increment in the efficacy determined by $\text{AUC}_0^{8\text{h}}$ and maximal intraocular pressure reduction. **Conclusion:** This work takes advantage of the high-affinity interaction between cationic carbosilane dendrimers and ocular transmembrane mucins, as well as the tensioactive behavior observed for these polymers. Our results indicate that low amounts of cationic carbosilane dendrimers are well tolerated and able

1 to improve the hypotensive effect of an acetazolamide solution. Our results suggest that
2
3 carbosilane dendrimers can be used in a safe range of concentrations to enhance the
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5 bioavailability of drugs topically administered in the eye.
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11 Keywords: Carbosilane dendrimers; ocular transmembrane mucins; mucoadhesion; surface
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13 plasmon resonance (SPR); hypotensive activity.
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Introduction

It is estimated that only about 5% of any drug administered by the topical ocular route is able to cross the cornea, reaching the anterior segment of the eye.¹ This makes it necessary to administer multiple doses of the drug per day. Furthermore, a portion of some administered drugs can be absorbed through the nasal and oral mucosa following drainage through the naso-lacrimal duct, and this can result in systemic side effects. One approach to increase ocular drug bioavailability is to maintain the formulation on the ocular surface longer. Classic (linear) bioadhesive polymers such as hyaluronic acid or cellulose derivatives included in eyedrop formulations can increase the drug residence time on the ocular surface. With this strategy, both the systemic drug side effects and the dosing frequency can be reduced.^{2,3} However, discomfort, blurred vision, and the presence of residual material around the eye are typically associated to these viscous media.

Recently, the use of dendritic structures has been explored with the aim of increasing the residence time of drugs on the ocular surface and reducing systemic absorption. For example, several authors have shown that polyamidoamine (PAMAM) dendrimers in solution⁴ or combined with polyethylene glycol as hydrogels⁵ can improve the residence time of several drugs. These dendritic structures can strongly interact with transmembrane ocular mucins, as recently demonstrated by our research group.⁶

The approach presented in this work uses new dendritic structures based on a C-Si backbone (carbosilane dendrimers) as potential bioadhesive polymers to be included in eyedrop formulations. These structures could benefit the administration of very poorly water-soluble drugs on the ocular surface. However, the higher hydrophobicity of carbosilane dendrimers implies that it is necessary to introduce ionic moieties (anionic or cationic groups) to achieve solubility in water.⁷⁻⁹ The objective of the present work is the evaluation of Generations 1, 2, and 3 of carbosilane dendrimers, with both anionic and cationic terminal groups, for topical ocular delivery. In an attempt to select the optimal concentration of these structures suitable for eye tissues, we first estimated the *in vitro* and *in vivo* tolerance in human

conjunctival and corneal cell lines and in rabbit eyes. Afterwards, the interaction of carbosilane dendrimers with transmembrane ocular mucin isolates was evaluated by surface plasmon resonance (SPR) spectroscopy.⁶ The most promising candidate was formulated with acetazolamide (ACZ), a hypotensive drug useful in glaucoma treatment after oral administration but which is inadequate for topical application due to low bioavailability after instillation. The eyedrop formulation was characterized and tested in normotensive rabbits.

Materials and Methods

Materials

We prepared anionic dendrimers $G_n\text{Si}(\text{CO}_2^-, \text{Na}^+)_m$ ($n = 1, m = 8$; $n = 2, m = 16$; $n = 3, m = 32$) as previously published.⁹ *t*-butyl N-allylcarbamate ($\text{C}_3\text{H}_5\text{NHBoc}$, Boc protected allylamine), Karstedt's catalyst, and HCl (Et_2O) were purchased from Sigma Chemical Co. (Madrid, Spain) and used as received.

A telomerase-immortalized, human corneal-limbal epithelial cell line (HCLE) was kindly supplied by Prof. I. K. Gipson (Schepens Eye Research Institute, Boston, MA, USA), and the human conjunctival epithelial cell line (IOBA-NHC) was provided by IOBA (Instituto de Oftalmología y Biología Aplicada, Valladolid, Spain). Reagents for cell cultures were purchased from Gibco[®] (Life Technologies, Barcelona, Spain). Acetazolamide, sodium chloride, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL in phosphate-buffered saline) were obtained from Sigma Chemical Co. (Madrid, Spain). Reagents and materials used for the SPR experiments were obtained from GE Healthcare (Orsay, France). All other chemicals used were reagent grade and used as received.

Animals

Male New Zealand white rabbits (San Bernardo Farm, Navarra, Spain), weighing 3-3.5 kg, were used for some experiments. The animals were kept in individual cages with free access to food and water. They

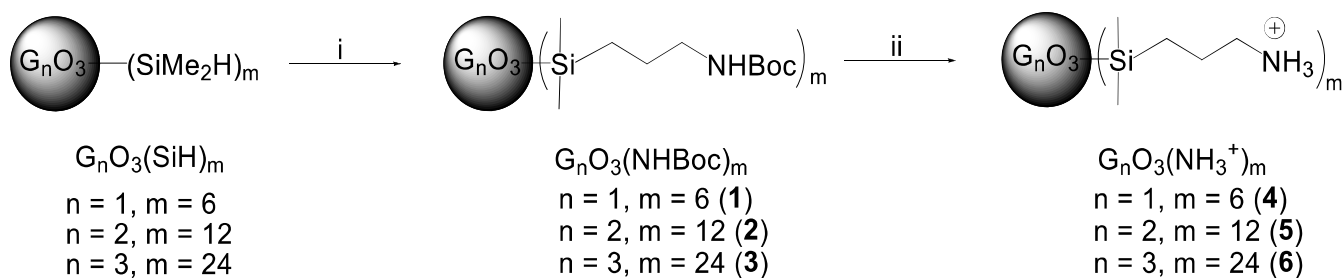
were housed in a room with controlled 12h/12h light/dark cycles and maintained at 22°C with 50% relative humidity. All of the protocols herein complied with the ARVO Statement for the Use of Animals in Ophthalmology and Vision Research and also are in accordance with the European Communities Council Directive (86/609/EEC).

Methods

Cationic dendrimers: preparation and characterization

All reactions were carried out under an inert atmosphere, and solvents were purified from appropriate drying agents when necessary. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity VXR-300 (300.13 (^1H), 75.47 (^{13}C) MHz) or on a Bruker AV400 (400.13 (^1H), 100.60 (^{13}C), 79.49 (^{29}Si) MHz). Chemical shifts (δ) were recorded in parts per million (ppm). ^1H and ^{13}C resonances were measured relative to internal deuterated solvent peaks considering TMS = 0 ppm. ^{29}Si resonances were measured relative to external TMS. When necessary, assignment of resonances was done from HSQC, HMBC, COSY, TOCSY, and NOESY NMR experiments. Elemental analyses were performed on a LECO CHNS-932 instrument. Mass Spectra were obtained from Bruker Ultraflex III and Agilent 6210 instruments. Starting dendrimers were $\text{G}_n\text{O}_3(\text{SiH})_m$ ($n = 1, m = 6$; $n = 2, m = 12$; $n = 3, m = 24$)¹⁰.

The synthesis of cationic dendrimers is presented in Scheme 1.



Scheme 1. Synthesis of cationic dendrimers $\text{G}_n\text{O}_3(\text{NH}_3^+)_m$ (Compounds **4-6**). (i) $\text{C}_3\text{H}_5\text{NHBoc}$, [Pt], 60 °C, 14 h; (ii) HCl (Et_2O).

G₁O₃(NHBoc)₆ (Compound 1) A solution of G₁O₃(SiH)₆ (0.500 g, 0.486 mmol), C₃H₅NHBoc (0.466 g, 2.966 mmol) and Karstedt's catalyst (3% mol of Si-H bonds) were stirred in dry THF (5 mL) in a Teflon-valved ampoule and heated at 50 °C for 16 h. Compound **1** was purified by size exclusion chromatography in THF. After removal of the solvent, Compound **1** was obtained as a pale yellow oil (0.681 g, 71%). For Compound **1**, the NMR data (δ, CDCl₃) was as follows: ¹H: -0.09 (s, 9 H, *Me*Si), -0.07 (s, 36 H, *Me*₂Si), 0.46 (m, 12 H, SiCH₂), 0.53 (m, 30 H, SiCH₂), 1.25 (m, 18 H, CH₂), 1.42 (m, 12 + 54 H, CH₂ and *t*-Bu), 1.75 (m, 6 H, OCH₂CH₂), 3.05 (m, 12 H, CH₂N), 3.87 (t, J = 6.3 Hz, 6 H, OCH₂), 4.56 (bs, 6 H, NH), 6.04 (s, 3 H, C₆H₃); ¹³C-{¹H}: -5.1 (*Me*Si), -3.4 (*Me*₂Si), 12.4 (NCH₂CH₂CH₂), 13.8 (SiCH₂), 18.4, 18.6, 20.0 (CH₂), 20.5 (OCH₂CH₂CH₂), 24.6 (CH₂CH₂N), 28.4 (*CMe*₃), 33.2 (OCH₂CH₂), 43.8 (NCH₂), 67.6 (OCH₂), 78.9 (*CMe*₃), 93.7 (C₆H₃ (CH), 155.9 (NHCO₂), 160.9 (*i*-C₆H₃); ²⁹Si: 1.8 (*Me*Si), 2.1 (*Me*₂Si). MS-ESI: 1988.3707 [M+NH₄]⁺. Elemental analysis for C₉₉H₂₀₄N₆O₁₅Si₉: Calcd. C, 60.31; H, 10.43; N, 4.26; Obt. C, 60.78; H, 10.72; N, 4.11.

G₂O₃(NHBoc)₁₂ (Compound 2) Following the above procedure, starting from G₂O₃(SiH)₁₂ (0.450 g, 0.210 mmol), C₃H₅NHBoc (0.400 g, 2.536 mmol), and Karstedt's catalyst, but washing with n-hexane after removal of volatiles, compound **2** was obtained as a pale yellow oil (0.668 g, 79%). For Compound **2**, the NMR (δ, CDCl₃) was as follows: ¹H: -0.11 (s, 27 H, *Me*Si), -0.07 (s, 72 H, *Me*₂Si), 0.43 (m, 26 H, CH₂Si), 0.53 (m, 72 H, CH₂Si), 1.27 (s, 24 H, CH₂), 1.42 (m, 24 + 108 H, CH₂ and *t*-Bu), 1.77 (m, 6 H, OCH₂CH₂), 3.05 (m, 24 H, CH₂N), 3.86 (m, 6 H, OCH₂), 4.59 (bs, 12 H, NH), 6.04 (s, 3 H, C₆H₃); ¹³C-{¹H}: -4.9(*Me*Si), -4.7 (*Me*Si), -3.3 (*Me*₂Si), 12.3 (SiCH₂), 13.8 (SiCH₂), 18.4, 18.6, 18.8, 20.0, 20.1 (CH₂), 20.6 (OCH₂CH₂CH₂), 24.6 (CH₂CH₂N), 28.4 (*CMe*₃), 33.3 (OCH₂CH₂), 43.6 (NCH₂), 67.6 (OCH₂), 78.9 (*CMe*₃), 93.7 (C₆H₃ (CH), 155.8 (NHCO₂), 160.9 (*i*-C₆H₃); ²⁹Si: 0.9 (*Me*Si), 1.8 (*Me*Si), 2.0 (*Me*₂Si). Elemental Analysis for C₂₀₁H₄₂₆N₁₂O₂₇Si₂₁: Calcd. C, 59.85; H, 10.65; N, 4.17; Obt. C, 60.15; H, 10.99; N, 4.03.

G₃O₃(NHBoc)₂₄ (Compound 3) Following the procedure described for Compound 2, starting from G₃O₃(SiH)₂₄ (0.450 g, 0.103 mmol), C₃H₅NHBoc (0.390 g, 2.474 mmol), and Karstedt's catalyst, Compound 3 was obtained as a pale yellow oil (0.728 g, 87%). For G₃, the NMR (δ, CDCl₃) data were as follows: ¹H: -0.11 (s, 54 H, MeSi), -0.07 (s, 144 H, Me₂Si), 0.45 (m, 48 H, SiCH₂), 0.52 (m, 174 H, SiCH₂), 1.27 (s, 48 H, CH₂), 1.42 (m, 48 + 216 H, CH₂ and *t*-Bu), 1.77 (m, 6 H, OCH₂CH₂), 3.05 (m, 24 H, CH₂N), 3.84 (m, 6 H, OCH₂), 4.63 (bs, 12 H, NH), 6.01 (s, 3 H, C₆H₃); ¹³C-{¹H}: -4.9 (MeSi), -3.3 (Me₂Si), 12.8 (CH₂CH₂CH₂N), 14.1 (SiCH₂), 17.9-20.6 (CH₂), 24.6 (CH₂CH₂N), 28.4 (CMe₃), 33.4 (OCH₂CH₂), 43.8 (CH₂N), 67.8 (OCH₂), 78.9 (CMe₃), 93.7 (C₆H₃ (CH), 155.9 (NHCO₂), 160.9 (*i*-C₆H₃); ²⁹Si: 0.9 (MeSi), 1.8 (MeSi), 2.1 (Me₂Si). Elemental Analysis for C₄₀₅H₈₇₀N₂₄O₅₁Si₄₅: Calc. C, 59.63; H, 10.75; N, 4.12; Obt. C, 59.00; H, 10.38; N, 3.09.

G₁O₃(NH₃Cl)₆ (Compound 4) A solution of Compound 1 (0.200 g, 0.100 mmol) in THF (10 mL) was treated with HCl (6.0 mL, 1 M in Et₂O) and stirred for 1 h. Afterward, the solution was filtered off and the residue was washed with Et₂O (2 x 25 mL) and dried, obtaining Compound 4 as a white solid (0.136 g, 85%). For Compound 4, the NMR (δ, D₂O) data were as follows: ¹H: -0.21 (s, 9 H, MeSi), -0.11 (s, 36 H, Me₂Si), 0.44 (s, 42 H, CH₂Si), 1.24 (s, 18 H, CH₂), 1.55 (s, 12 H, CH₂), OCH₂CH₂ not observed, 2.80 (s, 12 H, CH₂N), 3.55 (bs, 6 H, OCH₂), 5.69 (bs, 3 H, C₆H₃); ¹³C-{¹H}: -5.4 (MeSi), -3.9 (Me₂Si), 11.1 (NCH₂CH₂CH₂), 12.9 (SiCH₂), 18.4, 18.6, 20.0, 21.1 (CH₂), 22.1 (OCH₂CH₂CH₂), 31.9 (OCH₂CH₂), 48.0 (NCH₂), 66.6 (OCH₂), 93.1 (C₆H₃ (CH), 160.0 (*i*-C₆H₃); ²⁹Si: 1.9 (MeSi), 2.2 (Me₂Si). Elemental Analysis for C₆₉H₁₆₂Cl₆N₆O₃Si₉: Calc. C, 52.14; H, 10.27; N, 5.29; Obt. C, 53.01; H, 10.91; N, 5.10.

G₂O₃(NH₃Cl)₁₂ (Compound 5) Following the above procedure, starting from Compound 2 (0.200 g, 0.050 mmol) and HCl (12.0 mL, 1 M in Et₂O) and stirring for 2 h, Compound 5 was obtained as a white solid (0.132 g, 82%). For Compound 5, the NMR (δ, D₂O) data were as follows: ¹H: -0.18 (s, 99 H, MeSi and Me₂Si), 0.44 (s, 102 H, CH₂Si), 1.24 (s, 72 H, CH₂), 1.55 (s, 24 H, CH₂), OCH₂CH₂ not observed,

2.81 (s, 24 H, CH_2N), 3.55 (bs, 6 H, OCH_2), 5.69 (bs, 3 H, C_6H_3); $^{13}\text{C}-\{^1\text{H}\}$: -5.4 (*MeSi*), -3.9 (*Me}_2\text{Si}*), 11.2 ($\text{NCH}_2\text{CH}_2\text{CH}_2$), 12.9 (SiCH_2), 17.7, 18.8, 21.1 (CH_2), 22.1 ($\text{OCH}_2\text{CH}_2\text{CH}_2$), 31.5 (OCH_2CH_2), 48.0 (NCH_2), 66.6 (OCH_2), 93.1 (C_6H_3 (CH), 159.8 (*i*- C_6H_3); ^{29}Si : 1.0 and 1.7 (*MeSi*), 2.1 (*Me}_2\text{Si}*). Elemental Analysis for $\text{C}_{141}\text{H}_{342}\text{Cl}_{12}\text{N}_{12}\text{O}_3\text{Si}_{21}$: Calc. C, 51.80; H, 10.54; N, 5.14; Obt. C, 51.21; H, 10.17; N, 4.83.

$\text{G}_3\text{O}_3(\text{NH}_3\text{Cl})_{24}$ (Compound 6). Following the above procedure, starting from Compound 3 (0.200 g, 0.024 mmol) and HCl (12.0 mL, 1 M in Et_2O) and stirring for 2 h, Compound 6 was obtained as a white solid (0.134 g, 83%). For Compound 6, the NMR (δ , D_2O) data were as follows: ^1H : -0.16 (s, 63 H, *MeSi*), and *Me}_2\text{Si}*), 0.49 (bm, 270 H, CH_2Si), 1.29 (s, 138 H, CH_2), 1.55 (s, 48 H, CH_2), OCH_2CH_2 not observed, 2.86 (s, 48 H, CH_2N), OCH_2 not observed, C_6H_3 not observed; $^{13}\text{C}-\{^1\text{H}\}$: -4.4 (*MeSi*), -3.0 (*Me}_2\text{Si}*), 12.2 ($\text{NCH}_2\text{CH}_2\text{CH}_2$), 18.0-18.8, 19.9 (CH_2), $\text{OCH}_2\text{CH}_2\text{CH}_2$ not observed, OCH_2CH_2 not observed, 48.0 (NCH_2), OCH_2 not observed, C_6H_3 not observed, *i*- C_6H_3 not observed; ^{29}Si : 1.1 (*MeSi*), 2.1 (*Me}_2\text{Si}*). Elemental Analysis for $\text{C}_{285}\text{H}_{702}\text{Cl}_{24}\text{N}_{24}\text{O}_3\text{Si}_{45}$: Calc. C, 51.63; H, 10.67; N, 5.07; Obt. C, 51.01; H, 10.12; N, 4.67.

Carbosilane dendrimers of Generations 1 to 3, both anionic and cationic, were dissolved in NaCl 0.9% media prior to characterization. For simplicity, the nomenclature employed for dendrimers described in the remainder of this paper will be of the type “Gn-A” (for anionic dendrimers) or “Gn-C” (for cationic dendrimers), where “Gn” indicates the generation.

Tolerance studies

***In vitro* experiments** The cytotoxicity of saline solutions of C-Si anionic and cationic dendrimers G1-3 was assessed by the mitochondrial-dependent reduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to formazan (MTT method) according to previous works.³ The cytotoxicity studies were performed on immortalized human corneal-limbal epithelial (HCLE) cells, generously donated by Dr. Ilene K. Gipson (Schepens Eye Research Institute, Harvard Medical School,

Boston, MA, USA) and normal human conjunctiva (IOBA-NHC) cells generously donated by the Institute for Applied Ophthalmobiology (University of Valladolid, Valladolid, Spain).

HCLE cells and IOBA cells were seeded into 96-well culture plates (40,000 cells/well) and cultured at 37°C for 24 h in a 5% CO₂ atmosphere. Adherent cells were exposed to dendrimer solutions for 15 min (short-term), 1 h (intermediate-term), or 4 h (long-term). These times were chosen to emulate the types of dwell times on the ocular surface of various formulations. An isotonic NaCl solution with 0.005% benzalkonium chloride was used as a positive control.

Cell viability was determined by the mitochondrial reduction of MTT to formazan. The cells were incubated in a MTT solution (5 mg/mL in phosphate-buffered saline) for 3 h at 37 °C. After careful aspiration of the MTT solution, the cells were solubilized with dimethyl sulfoxide (100 µL/well). The extent of the reduction of MTT to formazan by the cells was quantified by optical density measurements at 550 nm using a plate reader (Digiscan mod 6010152EU, Eugendorf, Austria). Viability was set as 100% in untreated cells. Eight wells per sample were tested, and the assays were performed in triplicate. The results were expressed as the reduction in cellular viability compared to negative controls for at least three independent experiments.

***In vivo* experiments** The *in vivo* ocular tolerance studies of saline solutions containing C-Si anionic and cationic dendrimers G1-3 were performed by instillation of 25 µL of each solution onto the right eye in five male New Zealand albino rabbits. The contralateral eye received the same volume of saline solution (control). Examination of the eyes was performed by specular microscopy before instillation of any substance, just after instillation, and at two and six hours post-instillation. A slit lamp (SL-8Z, Topcon, Barcelona, Spain) was used to evaluate pupillary reflex, pupil size, superior and inferior eyelids, presence of redness, blepharitis and blepharospasm, tear charge, exudates, fluorescein tear film break up time (TBUT), redness of bulbar, limbal and tarsal conjunctival surfaces, inflammation of nictitating

membrane, and transparency of the cornea.¹¹ Ocular signs were graded using the Efron scale (0 = normal; 1 = trace; 2 = mild; 3 = moderate; 4 = severe)¹²

Mucoadhesion studies

After the tolerance studies, the interaction of selected concentrations of C-Si anionic and cationic dendrimers G1-3 with transmembrane ocular mucins was analyzed by SPR spectroscopy (Biacore[®] T100 GE Healthcare, Orsay, France). One substance, the ligand, is attached on the surface of a sensor chip. The second product, the analyte, is then pumped across the surface via a microfluidic system. If interaction between the ligand and the analyte occurs, the refractive index at the surface of the chip changes and is registered “on line” as an increase in signal.

Mucin isolation The isolation of transmembrane mucin from cultured HCLE cells has been previously described.⁶ Briefly, mucin was purified from stratified HCLE cultures by gel chromatography on a Sepharose CL-4B size exclusion column. The void volume containing the mucin fraction was digested with RNase A and DNase I and further purified by isopycnic density gradient centrifugation in cesium chloride.

Mucin immobilization In a preliminary step, the transmembrane ocular mucin isolates were immobilized on the Sia Kit Au chips (Biacore[®]) by incubation of the chip surface with 50 μ L of the transmembrane mucin solution isolated as described above (80 μ g/mL) overnight at room temperature. Afterwards, the chips were intensively rinsed in MilliQ[®] water. Once each chip was prepared and inserted on the Biacore[®] T100 apparatus, it was again extensively washed with MilliQ[®] water at 30 μ L/min for 12 h. Resonance units (RU) were measured before and after the immobilization step to monitor the amount of mucin attached to the Au chips. Calculations of immobilized mucin amounts were performed on the basis of the Biacore[®] standards where 1 RU = 1 μ g/mm². The optical mucin layer was calculated considering that 1 RU corresponds to a resonance angle shift of 10^{-4} degrees.¹³

Binding experiments Binding experiments were performed by flowing saline solutions of G1, G2, and G3 of C-Si anionic and cationic dendrimers (5 or 10 μM) over the mucin coated sensor chip surface within the Biacore[®] T100 apparatus for 300 s at 10 $\mu\text{L}/\text{min}$. After this contact step, the sensorgram (RU versus time) was collected until equilibrium was reached using physiological saline solution as the running medium control. All studies were performed in triplicate at 35°C, the physiological ocular surface temperature.¹⁴

Results were statistically analyzed by one-way analysis of variance (ANOVA) using SPSS version 16.0 software. Post-ANOVA analyses were carried out according to the Bonferroni's multiple comparisons test. Results were considered significant when $p < 0.05$.

Efficacy studies

Acetazolamide formulations: Preparation and characterization

Acetazolamide formulations were prepared according to the following procedures:

Formulation 1 (F1): ACZ 0.07% in saline solution A saturated solution of ACZ was prepared by dissolving 3.5 mg of the drug in 5 mL of 0.9% saline. The solution was stirred overnight at 30°C to ensure total dissolution.

Formulation 2 (F2): ACZ 0.07% in saline solution including 5 μM of G3-C carbosilane dendrimer A saturated solution of ACZ was prepared by dissolving 3.5 mg of the drug in 5 mL of 0.9% saline containing 5 μM of G3 cationic carbosilane. The solution was stirred overnight at 30°C to ensure total dissolution.

Both formulations and the saline solution (0.9%) were characterized in terms of surface tension, pH, and osmolarity. The pH measurements were performed at room temperature (25°C) with a pH-meter (model 230, Mettler, Barcelona, Spain) equipped with a microelectrode (InLab, Mettler). Osmolarity of the formulations and vehicle was measured with a Knauer vapor osmometer K-7000 (Knauer GmbH, Berlin, Germany). As a reference, a standard 400 mOsm solution of sodium chloride was used.

Determinations were performed at 33-35°C in triplicate. Surface tension of each formulation was determined by the Wilhelmy plate method (PL21; Krüss, Hamburg, Germany), with a digital tensiometer (model K11; Krüss, Hamburg, Germany) in combination with the integrated software (Laboratory Desktop software, ver. 3.11; Krüss, Hamburg, Germany) at 33-35°C. Before each measurement, the tensiometer was calibrated with MilliQ[®] water (72.0 ± 0.5 mN/m). The time required for equilibration of the formulations was set to 3 minutes. The formulations were assayed in triplicate, with three measurements for each replicate.

Intraocular pressure measurements

Intraocular pressure (IOP) was measured with a rebound tonometer (Icare Tonovet; Vantaa, Finland). For all of the preparations tested, the instillation volume was 25 μ L. Four animal groups were used for administration of Formulation 1 (18 right eyes), Formulation 2 (18 right eyes), saline solution 0.9% (10 right eyes), and saline solution 0.9% containing 5 μ M of G3-C cationic carbosilane dendrimer (10 right eyes). To study the time-course of the effect of F1 and F2, two IOP measurements were taken before any compound was administered (30 minutes and just before the instillation). Then, measurements were taken once every hour over a period of 8 hours.

The effect of each formulation was defined in terms of IOP reduction (Δ IOP). It was calculated using as reference the IOP basal (%) data determined before any instillation in both eyes for each rabbit. Data thus obtained underwent area under the curve-time curve (AUC, %, per hour) analysis according to the trapezoidal rule.

Data were expressed as the means \pm standard errors of the mean. Statistical differences between the compared values were evaluated by two-tailed student's t-test. P-values less than 0.05 were considered significant.

For efficacy studies, a comparison of the confidence interval was performed for several activity parameters: maximal IOP reduction and area under the curve of the IOP variation *versus* time plot.

Differences in the efficacy treatment between F1 and F2 were considered significant when the two-sided 95% confidence interval for the difference between the means of the selected parameters excluded zero.¹⁴

Results

Carbosilane dendrimers

Formation of cationic carbosilane dendrimers with -NH_3^+ moieties at the periphery can be achieved by protonation of neutral primary amine dendrimers.^{7,10} However, the synthesis of these amine derivatives requires harsh reaction conditions, and the amine dendrimers also have a high aggregation tendency, making storage and manipulation difficult. Furthermore, allylamine is a highly toxic and volatile liquid. For that reason, we used a different approach to obtain cationic carbosilane dendrimers.

First, neutral dendrimers were prepared with primary amino groups protected with Boc, $\text{G}_n\text{O}_3(\text{NHBoc})_m$ [$n = 1, m = 6$ (Compound **1**); $n = 2, m = 12$ (Compound **2**); $n = 3, m = 24$ (Compound **3**)] (Scheme 1). These compounds were obtained by a simple hydrosilylation reaction of commercially available $\text{C}_3\text{H}_5\text{NHBoc}$. This material is a solid that is clearly lesser toxic than allylamine, and it has dendrimers that can be decorated with Si-H terminal bonds following a procedure similar to that described for related carbosilane dendrons.¹⁵ The neutral dendrimers thus obtained can be easily stored even in the absence of solvents. NMR spectroscopy showed the disappearance of resonances corresponding to the starting SiMe_2H functions and the presence of the new groups. The new alkyl chain-formed $\text{Si}(\text{CH}_2)_3\text{N}$ was detected by TOCSY-NMR spectroscopy, observing the three methylene groups as multiplets at about 3.0 ppm (CH_2N), 1.4 (internal CH_2), and 0.5 (CH_2Si). By ^{13}C NMR spectroscopy, ^{13}C was found at about 44 ppm. Also, the carbonyl carbon atom was present at about 156 ppm. Finally, in the ^{29}Si NMR spectra, the outermost silicon atom was present at about 2.3 ppm, whereas the initial SiH resonance was observed at about -14 ppm.

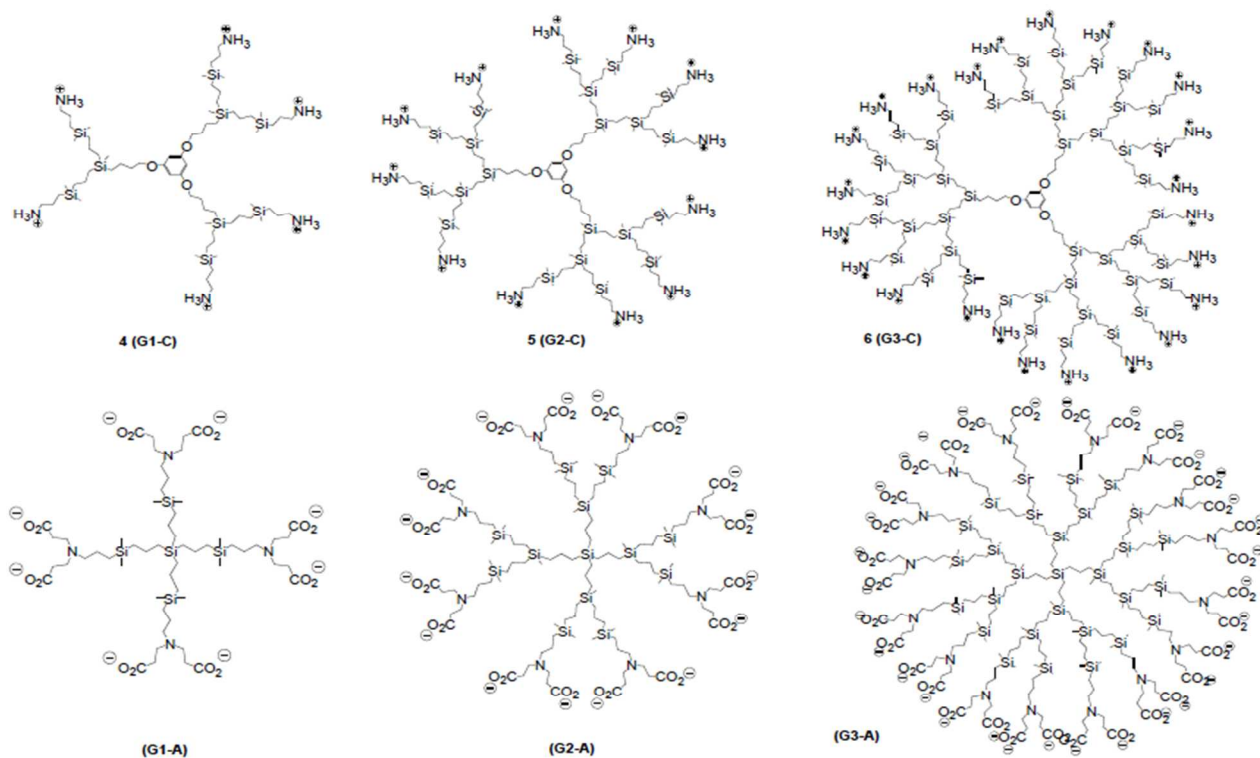


Figure 1: Drawing of cationic Gn-C (Compounds 4-6) and anionic dendrimers Gn-A used in this work

After formation of the cationic carbosilane dendrimers, deprotection of primary amines was successfully performed by addition of excess HCl, isolating the new cationic derivatives $\text{G}_n\text{O}_3(\text{NH}_3^+)_m$ [$n = 1, m = 6$ (Compound 4); $n = 2, m = 12$ (Compound 5); $n = 3, m = 24$ (Compound 6)] (denoted as G1-C, G2-C and G3-C in this paper) (Scheme 1). These derivatives appeared as white solids that were soluble in water, DMSO, and MeOH. In these compounds, the main NMR resonance belonged to the CH_2N groups, observed at about 2.80 ppm in the ^1H NMR spectra and at about 48.0 ppm in the ^{13}C NMR spectra. The final structures are presented in Figure 1.

In vitro tolerance studies

The exposure of HCLE and IOBA-NHC cultures to the anionic and cationic C-Si dendrimers for 15 minutes was intended to mimic a single drop administration to the ocular surface. Exposures for 1 and 4 h were designed to simulate repeated exposures to the ocular surface.

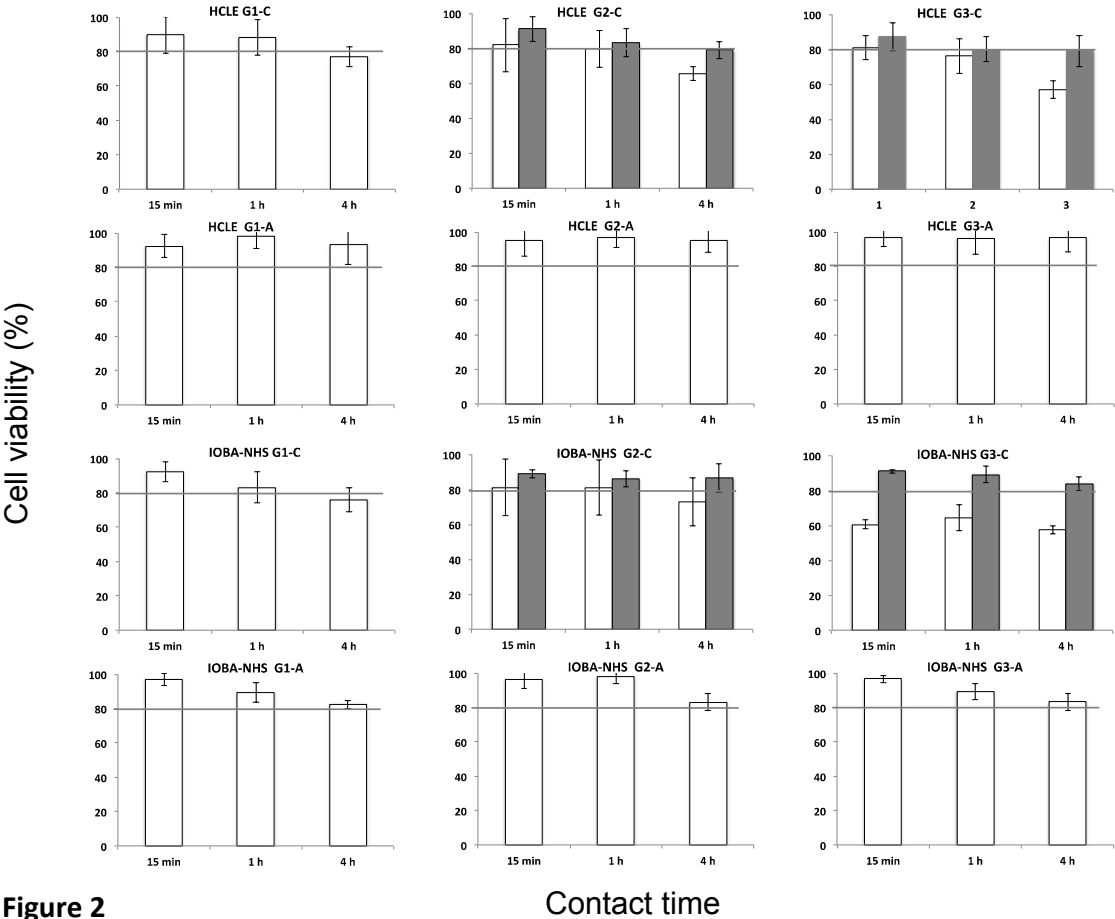


Figure 2

Contact time

Figure 2: *In vitro* tolerance of the HCLE and IOBA-NHC cell lines to cationic and anionic C-Si dendrimers (Gn-C and Gn-A) in saline solution. White bars, 10 μM; grey bars, 5 μM; n, generation number.

All three generations of the anionic dendrimers and generations 1 and 2 of the cationic dendrimers were well tolerated at 10 μM (Figure 2). The compounds with percentages of cell survival higher than 80% relative to non-treated cultures of both cell lines were further evaluated. For the third generation of

carbosilane cationic dendrimers, G3-C, it was necessary to reduce the concentration to 5 μM to obtain non-toxic solutions.

In vivo tolerance studies

At 5 and 10 μM , the evaluated carbosilane dendrimer solutions presented neither discomfort nor abnormal clinical signs after topical administration in rabbit eyes (Table 1). The Efron scale scores ranged from 0 – 2 in all cases.

Table 1: *In vivo* tolerance studies in albino rabbit eyes after instillation of 25 μL saline solutions of C-Si dendrimers of generation G1, G2, and G3 bearing anionic or cationic surface groups.

Before instillation					
Preparation	Tearing	Blepharospasm	Limbal	Tarsal	Cornea*
			Conjunctiva*	Conjunctiva*	
G1A (10 μM)	No	No	1-2	0-1	1
G2A (10 μM)	No	No	1-2	0-1	1
G3A (10 μM)	No	No	1-2	1	1
G1C (10 μM)	No	No	1-2	1	0-1
G2C (10 μM)	No	No	1-2	1	0-1
G3C (5 μM)	No	No	1	1	0-1
Saline solution	No	No	1-2	1	0-1
(left eyes)					
Just after instillation					
Preparation	Tearing	Blepharospasm	Limbal	Tarsal	Cornea*
			Conjunctiva*	Conjunctiva*	
G1A (10 μM)	No	No	1-2	0-1	1
G2A (10 μM)	N	No	1-2	0-1	1
G3A (10 μM)	No	No	1-2	1	1
G1C (10 μM)	No	Slight	2	1	0-1

G2C (10 µM)	No	No	2	1	0-1
G3C (5 µM)	No	No	1	1	0-1
Saline solution	No	No	1-2	1	0-1
(left eyes)					
2h post-instillation					
			Limbal	Tarsal	
Preparation	Tearing	Blepharospasm	Conjunctiva*	Conjunctiva*	Cornea*
G1A (10 µM)	No	No	1	0-1	1
G2A (10 µM)	No	No	1	0-1	1
G3A (10 µM)	No	No	1-2	1	1
G1C (10 µM)	No	No	0-1	1	0-1
G2C (10 µM)	No	No	0-1	1	0-1
G3C (5 µM)	No	No	1-2	1	0-1
Saline solution	No	No	1-2	1	0-1
(left eyes)					
6h post-instillation					
			Limbal	Tarsal	
Preparation	Tearing	Blepharospasm	Conjunctiva*	Conjunctiva*	Cornea*
G1A (10 µM)	No	No	1	0-1	0-1
G2A (10 µM)	No	No	1	0-1	0-1
G3A (10 µM)	No	No	1-2	1	0-1
G1C (10 µM)	No	No	1	0-1	0-1
G2C (10 µM)	No	No	1	0-1	0-1
G3C (5 µM)	No	No	1-2	1	0-1
Saline solution	No	No	1-2	1	0-1
(left eyes)					

*, numeric scores based on the Efron scale: 0 = normal; 1 = trace; 2 = mild; 3 = moderate; 4 = severe

Interaction of carbosilane dendrimers with transmembrane ocular mucins

The carbosilane dendrimer concentrations used to assess the interaction between the dendrimers and transmembrane ocular mucins were the same as used in the *in vivo* tolerance studies. In these experiments, the behaviors of the anionic and cationic dendrimers in contact with ocular mucin were completely different from one another. The sensorgram values for the anionic dendrimers returned to almost zero after about 350 s, indicating that the interaction between the dendrimers and mucin was non-permanent (Figure 3a). In contrast, a significant portion of the cationic dendrimers in contact with the ocular mucin layer was retained in the glycoprotein layer, permanently modifying the RU signal (Figure 3b). The highest retention was observed for 5 μ M G3-C.

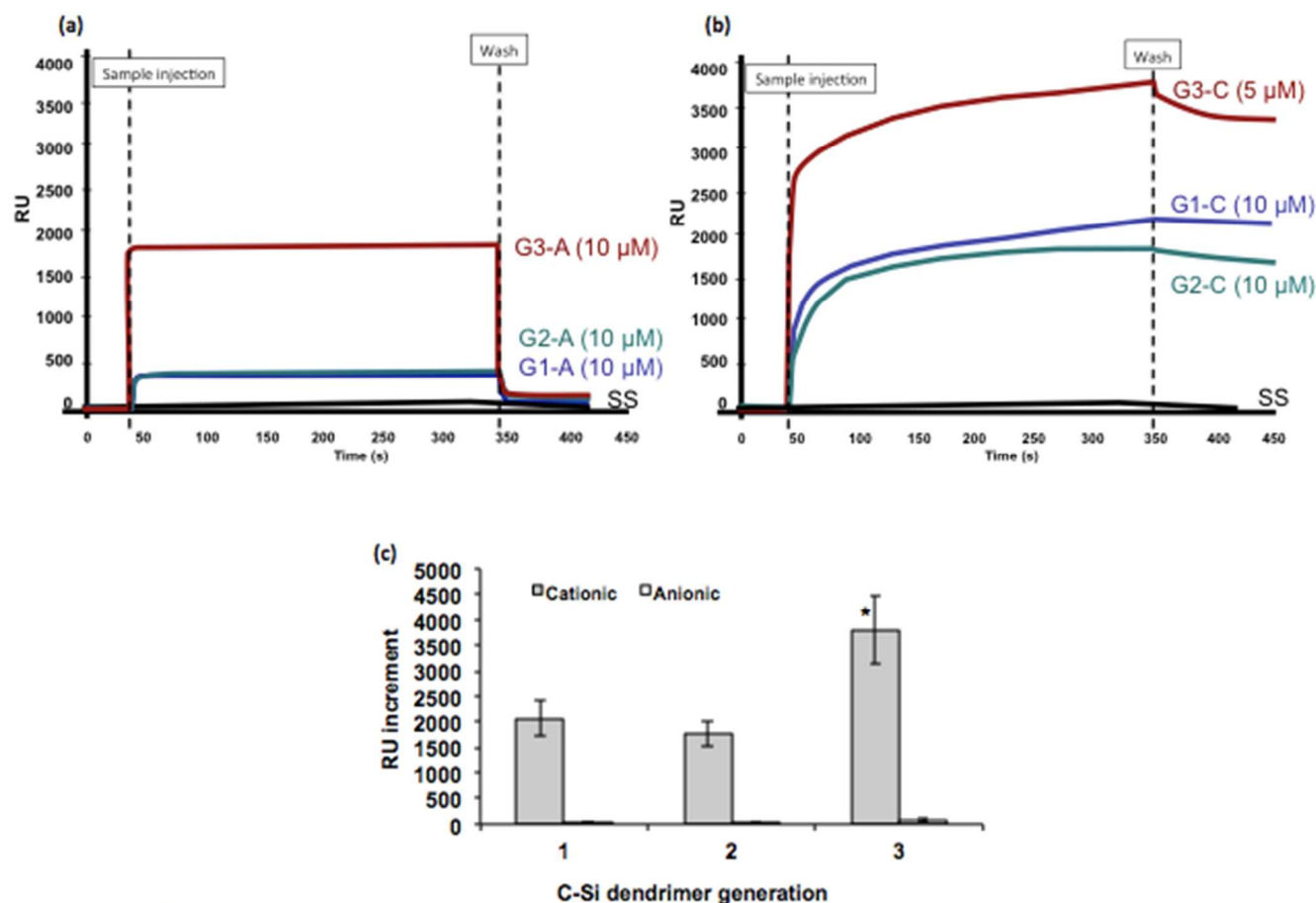


Figure 3

Figure 3: Dendrimer-transmembrane ocular mucin interaction measured by SPR spectroscopy. **(a)** Binding sensorgram of interaction between ocular transmembrane mucin and Generations 1 – 3 of anionic carbosilane dendrimers. **(b)** Binding sensorgram of interaction between ocular transmembrane mucin and Generations 1 – 3 of cationic carbosilane dendrimers. **(c)** Comparison of total cationic and anionic dendrimer binding, measured as RU increments, for each of the three generations of Gn-C and Gn-A dendrimers. RU, resonance units; SS, saline solution *, $p < 0.05$ for comparison of cationic dendrimer Generation 3 with cationic dendrimer Generations 1 and 2.

Formulation characterization: pH, surface tension, and osmolarity

Based upon the mucoadhesion studies, the most promising candidate dendrimer was 5 μ M G3-C, which we then used to prepare the ACZ formulation (drug:polymer ratio 3149:1). The pH, surface tension, and osmolarity of the ACZ solution in physiological saline media (0.9%), with and without dendrimers (F2 and F1 respectively), were determined (Table 2).

Table 2: ACZ formulation pH, osmolarity and surface tension.

ACZ formulation	pH	Osmolarity (mOsm)	Surface tension (mN/m)
F1	5.6 \pm 0.1	294.5 \pm 1.5	54.7 \pm 0.7
F2	5.6 \pm 0.1	289.4 \pm 2.1	41.7 \pm 0.2

F1, 0.07% ACZ in 0.9% NaCl; F2, 0.07% ACZ in a solution of 5 μ M G3-C dendrimers and 0.9% NaCl. Data are expressed as means \pm SEMs (n = 3).

The pH of both F1 and F2 was 5.6 \pm 0.1 (Table 2). The osmolarity of the F1 formulation was 294.5 \pm 1.5 mOsm, which was not significantly different from the F2 osmolarity (Table 2). The surface tension of F2 was significantly lower than F1 ($p < 0.05$), reflecting moderate tensioactive behavior for G3-C dendrimers.

Intraocular pressure

Several activity parameters were selected to evaluate the effect of 5 μ M G3-C dendrimer on the ACZ-induced hypotension: onset time, peak effect time, maximal IOP reduction (Δ IOP_{max}), effective time period, and area under the IOP% *versus* time curve (AUC₀^{8h}). Neither 0.9% saline (Figure 4a) nor 0.9% saline with 5 μ M G3-C dendrimer (Figure 4b), both of which served as ACZ-free controls, had a hypotensive effect. For F1, composed of 0.07% ACZ in 0.9% saline, the onset of hypotension was evident at 2 h post-instillation and lasted for four hours ($p < 0.05$, Figure 4a). For F2, composed of 0.07% ACZ in 0.9% saline containing 5 μ M G3-C dendrimer, the hypotensive onset was evident at 1 h post-instillation and lasted for 7 hours ($p < 0.05$, Figure 4b). Furthermore, the presence of the dendrimer in the formulation slightly delayed the maximal Δ IOP effect.

The maximum hypotensive effect, 22.6%, was reached by F2, and it was significantly greater than the value achieved by F1, 17.2% ($p = 0.0057$, Figure 4c). The AUC₀^{8h} of F1, 72.8% h, was lower than that for F2 ($p = 0.0137$, Figure 4d). The efficacy of the formulations, as measured by the confidence intervals of the AUC₀^{8h} and the Δ IOP_{max}, was significantly higher for F2 than F1 (Figure 5).

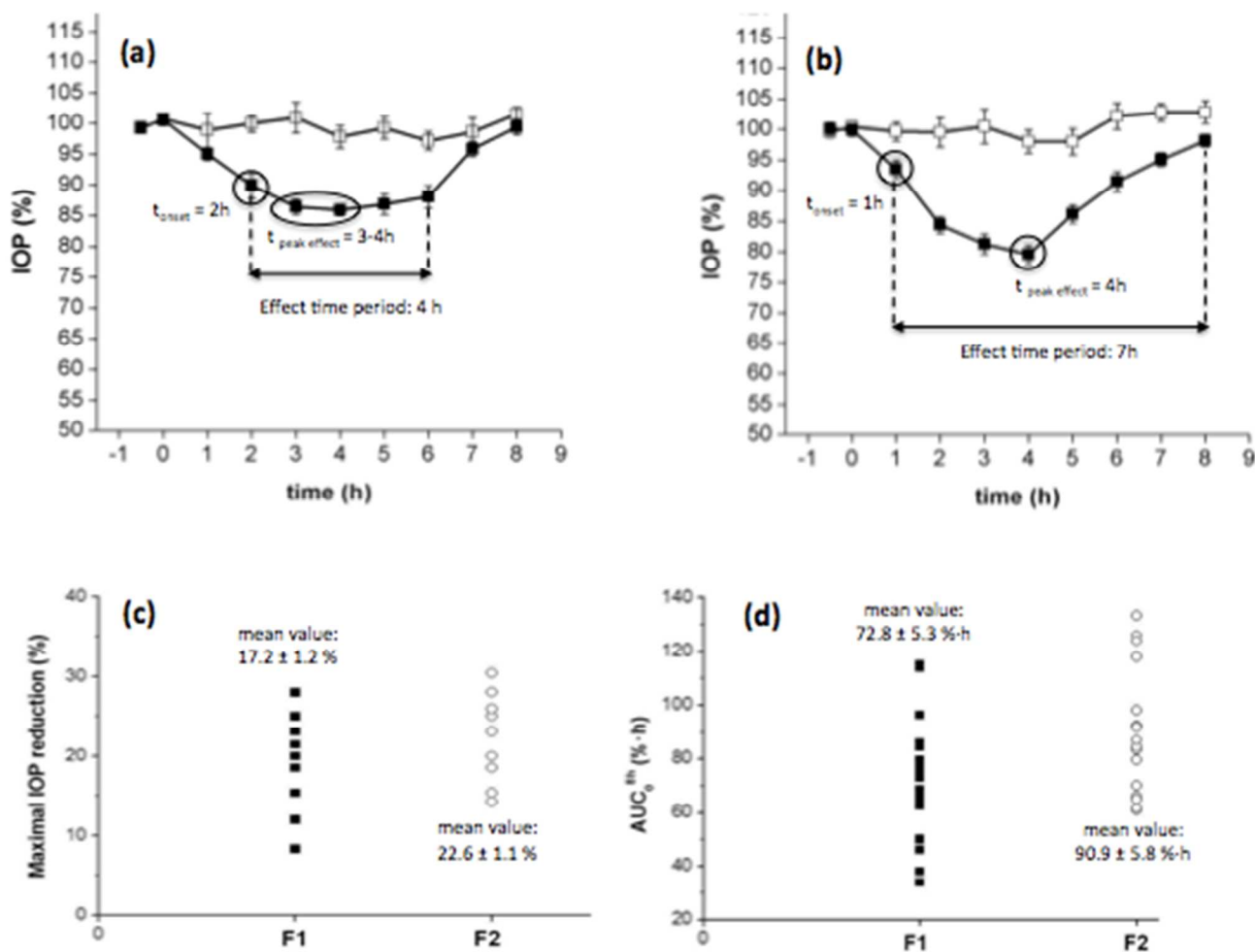


Figure 4

Figure 4: (a) IOP after instillation of 25 μL of F1 composed of 0.07% ACZ in 0.9% saline (black squares) or the vehicle composed of 0.9% saline (white squares). (b) IOP after instillation of 25 μL of F2 composed of 0.07% ACZ in 0.9% saline containing 5 μM G3-C dendrimer (black squares), and the vehicle composed of 0.9% saline containing 5 μM 3G-C dendrimer (white squares). (c) Maximal IOP reduction (ΔIOP_{max} , %) induced by F1 and F2. (d) Area under the curve of the $\Delta IOP(\%)$ versus time (h) from 0 to 8 hours (AUC_0^{8h}) induced by F1 and F2.

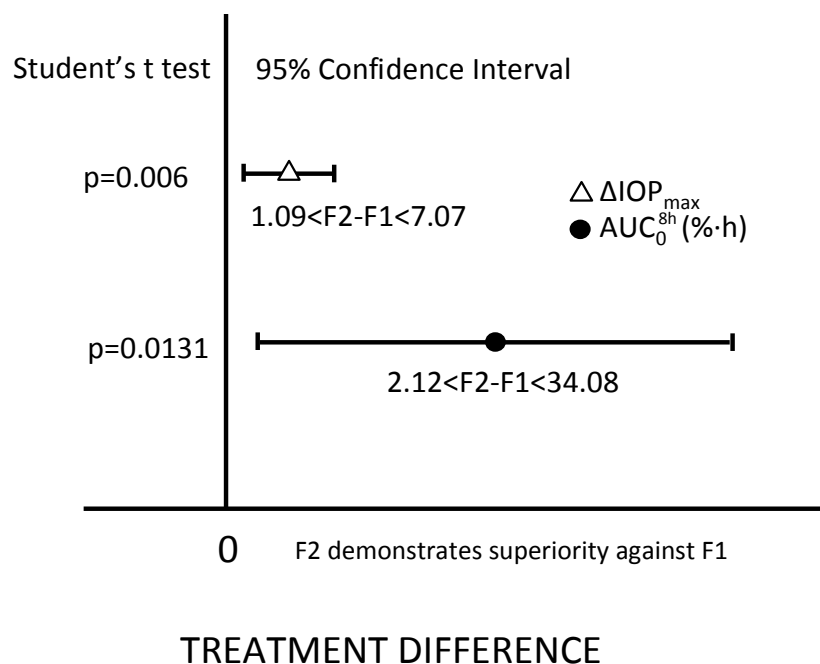


Figure 5: Relationship between the two mean difference test (Student's t-test) and confidence intervals for the difference of the means. Values to the right of zero correspond to a better response to F2 than to F1 for both AUC_0^{8h} and ΔIOP_{\max} . F1, 0.07% ACZ in 0.9% saline; F2, 0.07% ACZ in 0.9% saline containing 5 μ M G3-C dendrimer.

Discussion

Dendrimers offer very attractive features as novel drug nanocarrier systems. These features include a controlled and totally known chemical structure and charge as well as the possibility of interaction with biological tissues and drugs.¹⁶⁻¹⁹ However, their use in ophthalmology remains almost unexplored in contrast to other administration routes (oral, intravenous, or transdermal) where extensive progress has been achieved.^{20,21} Only a few authors have explored the potential of dendrimers as drug carriers in

ophthalmology. Vandamme and Brobeck demonstrated that the co-administration of PAMAM dendrimers with pilocarpine or tropicamide prolonged and sustained the pharmacological effect of the drugs in comparison to classical drug solutions.⁴ More recently, using surface biosensors we demonstrated a strong interaction between PAMAM dendrimers and ocular transmembrane mucins.⁶ According to these initial works, it is worth considering dendrimers as useful tools to increase drug retention time on the ocular surface, which in turn could reduce the administration frequency and thus increase patient compliance. With this idea, we generated new dendrimers based on a carbosilane structure, and evaluated the suitability as eyedrop excipients. The carbosilane skeleton provides these dendrimers with unique advantages. Among them, a simple method for synthesis, chemical inertia of the carbon-carbon and carbon-silicon bonds that prevent secondary reactions, and importantly, a highly hydrophobic character that allows better interaction of the dendrimer with biological membranes and/or hydrophobic drugs.

Because this was the first time that these structures have been evaluated for ocular administration, we performed tolerance studies to determine the suitable concentration for ocular tissues. The initial *in vitro* tolerance assay with human ocular cell lines showed higher toxicity for the cationic dendrimers than for the anionic ones. These results are in concordance with other cytotoxicity studies performed in different cell lines.^{22,23} Some authors have attributed the cytotoxicity of cationic dendrimers to the high density of positive charges that increases with each generation of dendrimer and that could disrupt the cell membranes.²⁴⁻²⁶ Nevertheless, several studies have demonstrated that cationic dendrimers have the same or lower toxicity than polycationic linear polymers.²⁶⁻²⁸ Indeed, Vandamme and Brobeck, for example, observed better tolerance results for PAMAM dendrimers than for linear polymers (hydroxypropylmethyl cellulose and Carbopol[®]) on the ocular surface.⁴ Furthermore, Bermejo and co-workers evaluated the biocompatibility of carbosilane dendrimers in primary cell cultures of peripheral blood mononuclear cells and erythrocytes and found they had good toxicity profiles over extended periods of time.⁷ Based upon our *in vitro* tolerance studies, we administered anionic and cationic carbosilane dendrimer solutions to the

ocular surface of rabbit eyes and found no alterations or discomfort. This further confirmed that the polymer concentrations selected in the *in vitro* study were suitable for ocular administration in animals.

We next determined the possible interaction of carbosilane dendrimers with elements of the ocular surface, specifically with corneal transmembrane mucins. These high molecular weight glycoproteins cover the corneal and conjunctival epithelia, anchor the precorneal tear film, and play an essential role in mucoadhesion.²⁹ We used surface biosensors with high sensitivity, so only small amounts of ligand and analyte were needed.³⁰⁻³² Because this is the first *in vitro* method specifically designed to evaluate interfacial mucin-polymer interactions, we recently proposed it as a very valuable tool in understanding the interactions of ocular transmembrane mucins with polymers or other macromolecules, especially when low amounts of mucins are available.⁶

The cationic dendrimer G3-C exhibited a significant permanent chemical adherence to the ocular transmembrane mucins. This interaction might be governed by electrostatic interactions between primary amino groups of the dendrimers and the sialic acid and sulphated groups present in the glycosylated chains of the mucins. In a previous work, this technique showed that the anionic dendrimer PAMAM-COOH permanently interacted with the ocular mucin layer, probably due to the formation of hydrogen bonds between charged carboxylic groups of the dendrimer and the sugar residue on the mucins side chains.⁶ However, the anionic carbosilane dendrimers used in the current work had a more hydrophobic backbone and non-protonated terminal carboxylate groups compared to PAMAM. Thus the formation of such interactions between the anionic carbosilane dendrimers and the mucins was not favored, probably because the electrostatic repulsion with the negatively charged mucin surface was strong enough to prevent permanent chemical interaction. Due to the lack of mucin interaction, we chose not to pursue the anionic dendrimers in further studies.

ACZ was chosen as the model drug to evaluate the potential of enhancing ocular surface bioavailability. This carbonic anhydrase inhibitor has a potent specific effect in reducing aqueous humor

production, and therefore, intraocular pressure. It has high potential in the treatment of glaucoma, in which high and persistent IOP promotes damage of the optic disk at the juncture of the optic nerve and retina, provoking irreversible retinal degeneration and blindness. However, the use of ACZ is limited because it has low bioavailability after ocular instillation due to low aqueous solubility and limited ocular penetration. For those same reasons, ACZ has gained attention as a model drug to test different strategies to increase bioavailability after ophthalmic application. Some efforts have been explored with this model drug, from the classical approach of using drug suspensions in polymeric solutions³³ to the use of more structured and sophisticated systems such as liposomes,³⁴ cyclodextrin complexes,^{35,36} niosomes,³⁷ nanoparticles,³⁸ and commercial poly(propyleneimine) dendrimers.³⁹ In those studies, ACZ efficacy was improved; however in some cases, tolerance studies were either not performed or they were unsuitable. Consequently, the potential cytotoxicity of the formulations is unknown.

In this work, we performed the essential *in vitro* and *in vivo* tolerance studies that allowed us to select an innocuous final prototype for *in vivo* efficacy studies. pH and osmolarity studies confirmed that the behavior of the selected formulation is totally compatible with the ocular surface.⁴⁰ The very simplistic approach explored takes advantage of the high interaction of cationic carbosilane dendrimers with ocular transmembrane mucins as well as the tensioactive behavior observed for these polymers. The inclusion of a very low amount of cationic carbosilane dendrimer, 5 μ M G3-C, in the ACZ solution in saline media resulted in a more rapid and extended hypotensive effect, leading to a significant increment in the efficacy. The low drug:polymer ratio used in this formulation indicates that most of the drug must not be directly attached to the dendrimer structure. Under these conditions, the inclusion of the cationic carbosilane dendrimers might promote the formation of a well-spread aqueous eyedrop layer, favored by the tensioactive nature of the formulation, that is strongly attached to the ocular surface and that enhances the retention of the drug. In contrast to formulations containing linear polymers, this increment in the

retention time would be achieved without modifying the viscosity of the tear film, preventing the discomfort typically associated to these viscous media.²⁹

Conclusions

The high chemical versatility and the small and controlled size of dendrimers allow the design of preparations able to interact with biological molecules, representing a simplified alternative to more complex systems for drug administration. In this work, we evaluated the potential use of ionic C-Si dendrimers for ocular administration. *In vitro* and *in vivo* tolerance studies were performed to select an innocuous prototype.

In this proof of concept, the inclusion of only a very low amount of cationic carbosilane dendrimer was well tolerated and improved the hypotensive effect of an ACZ solution by decreasing the onset time and increasing the duration after a single instillation. This is the first work demonstrating that carbosilane dendrimers can enhance the bioavailability of drugs administered topically in the eye.

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4 Eye Institute Grant No. R01EY014847 (PA).
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9 **Notes and references**
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14 Electronic Supplementary Information (ESI) available: Drawing and NMR spectra of compounds
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16 described in the paper.
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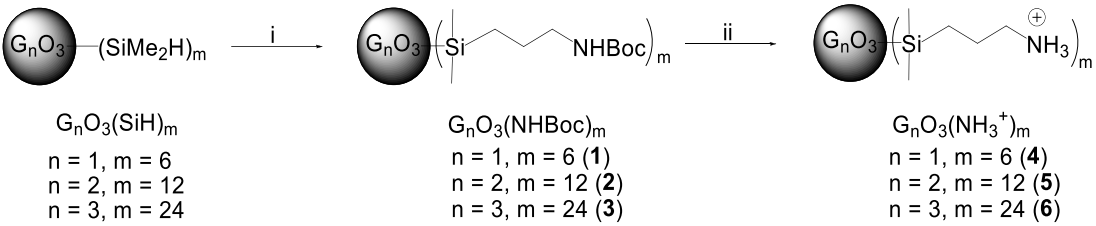
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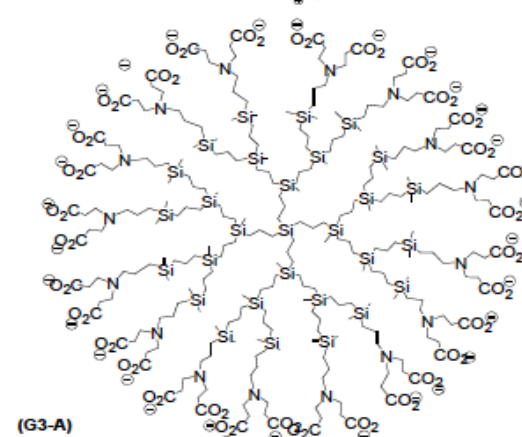
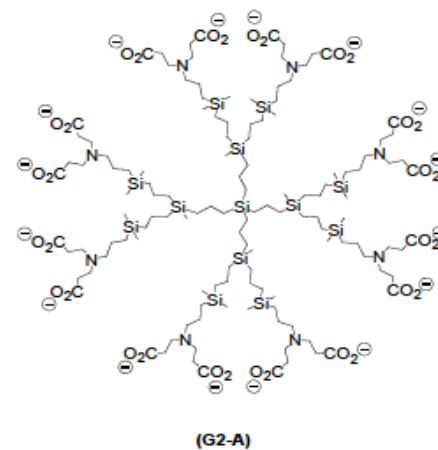
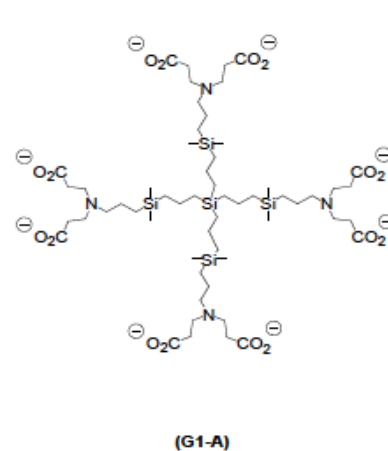
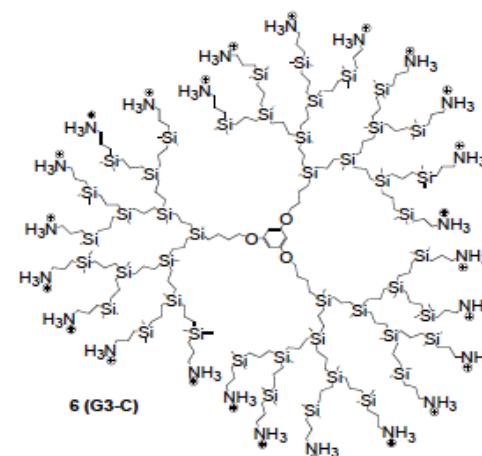
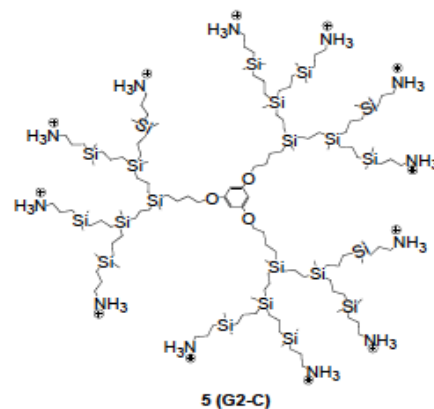
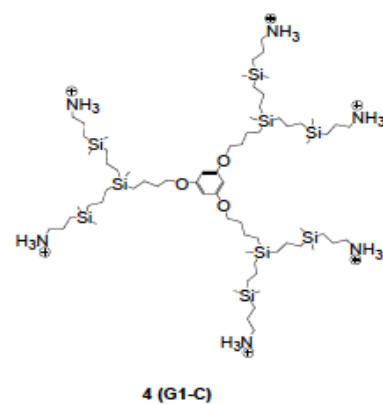
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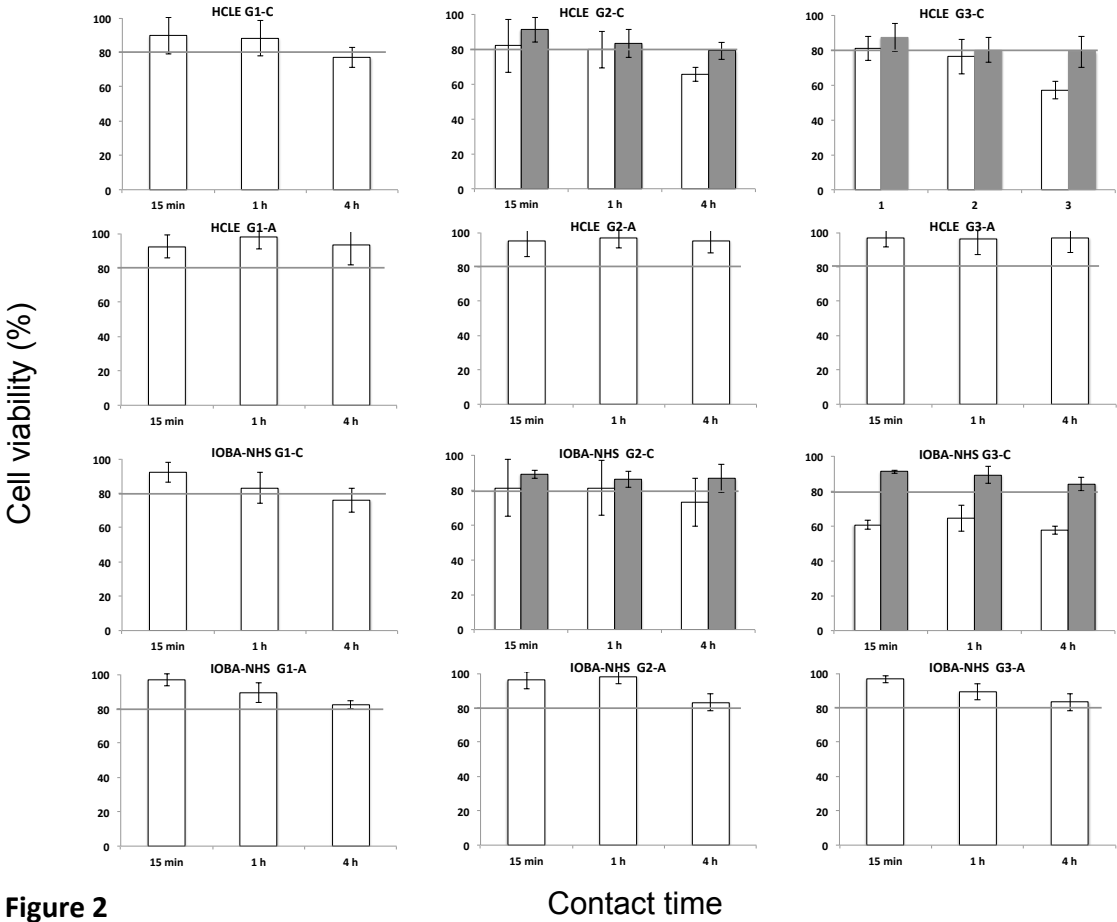
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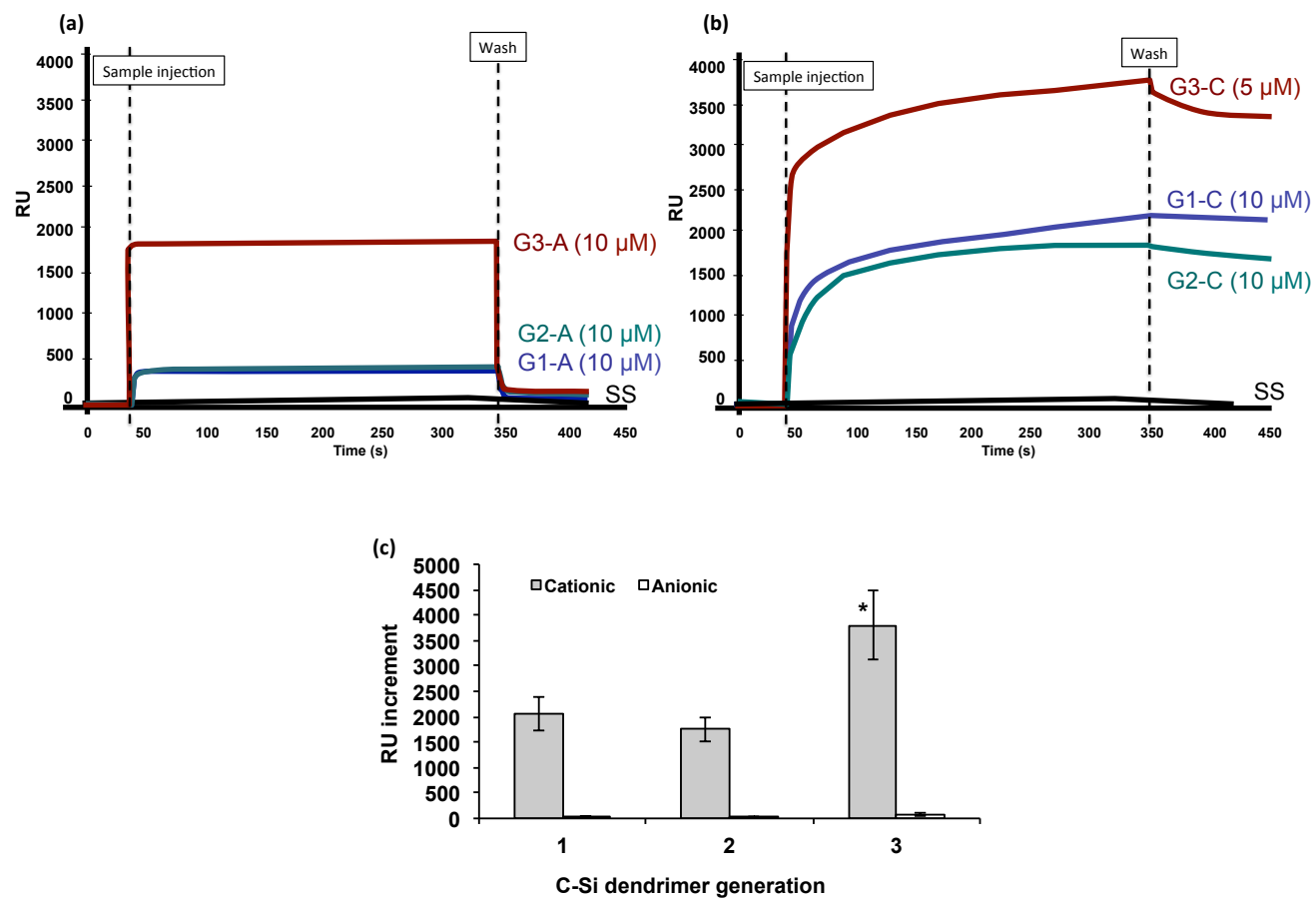


Figure 3

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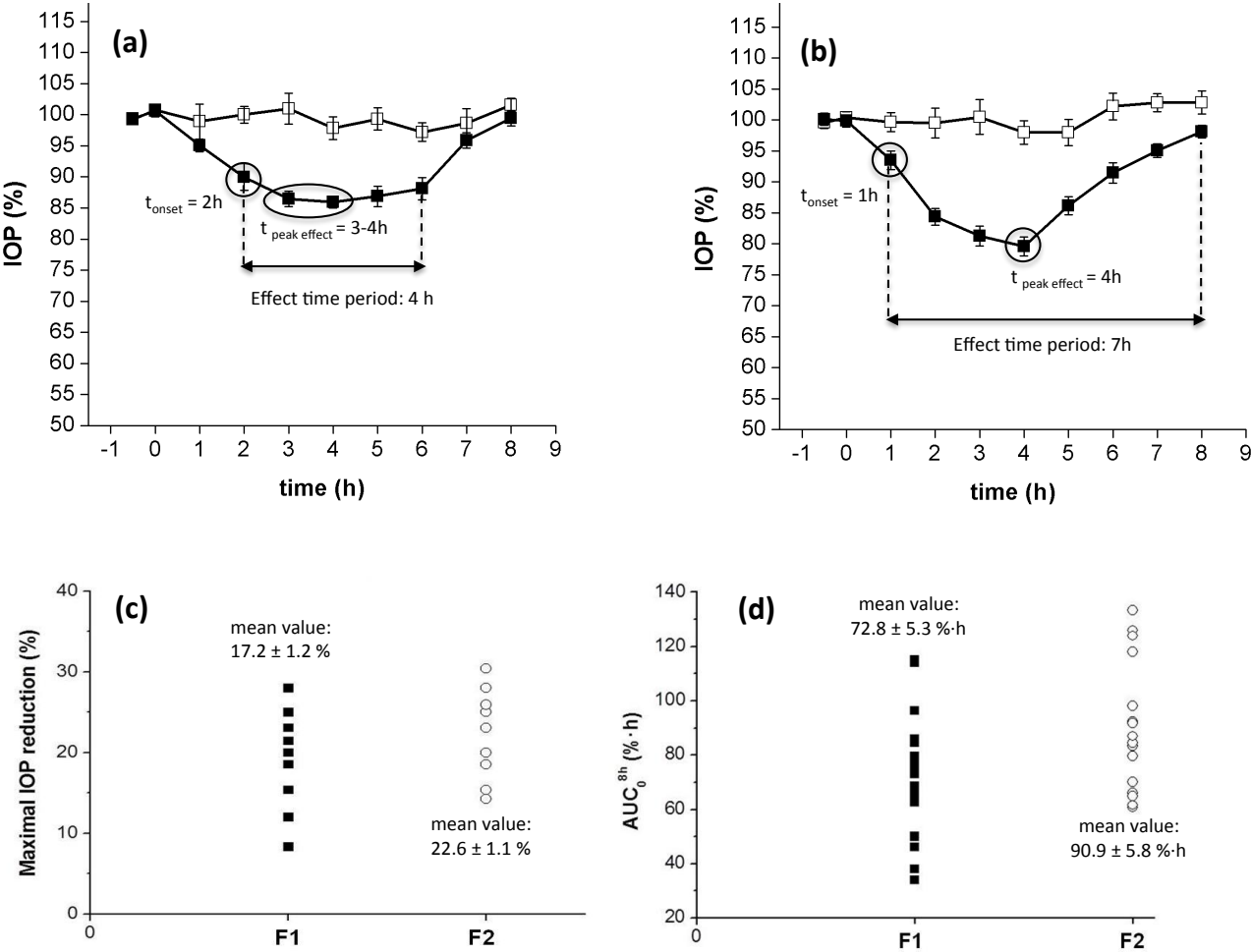


Figure 4

