Pharm Res (2012) 29:2329–2340
DOI 10.1007/s11095-012-0761-1

RESEARCH PAPER

Interfacial Interaction between Transmembrane Ocular Mucins and Adhesive Polymers and Dendrimers Analyzed by Surface Plasmon Resonance

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Received: 17 December 2011 / Accepted: 17 April 2012 / Published online: 8 May 2012

ABSTRACT

Purpose Development of the first *in vitro* method based on biosensor chip technology designed for probing the interfacial interaction phenomena between transmembrane ocular mucins and adhesive polymers and dendrimers intended for ophthalmic administration.

Methods The surface plasmon resonance (SPR) technique was used. A transmembrane ocular mucin surface was prepared on the chip surface and characterized by QCM-D (Quartz Crystal Microbalance with Dissipation) and XPS (X-ray photoelectron spectroscopy). The mucoadhesive molecules tested were: hyaluronic acid (HA), carboxymethyl cellulose (CMC), hydroxypropylmethyl cellulose (HPMC), chitosan (Ch) and polyamidoamine dendrimers (PAMAM).

Results While Ch originated interfacial interaction with ocular transmembrane mucins, for HA, CMC and HPMC, chain interdiffusion seemed to be mandatory for bioadherence at the concentrations used in ophthalmic clinical practise. Interestingly, PAMAM dendrimers developed permanent interfacial interactions with transmembrane ocular mucins whatever their surface chemical groups, showing a relevant importance of co-operative effect of these multivalent systems. Polymers developed interfacial interactions with ocular membrane-associated mucins in the following order: Ch(1 %) > G4PAMAM-NH_2(2 %) = G4PAMAM-OH(2 %) > G3.5PAMAM-COOH(2 %)>> CMC(0.5 %) = HA(0.2 %) = HPMC(0.3 %).

Conclusions The method proposed is useful to discern between the mucin-polymer chemical interactions at molecular scale. Results reinforce the usefulness of chitosan and dendrimers as polymers able to increase the retention time of drugs on the ocular surface and hence their bioavailability.

KEY WORDS chitosan · mucoadhesion · ocular transmembrane mucins · polyamidoamine (PAMAM) dendrimers · surface plasmon resonance (SPR)

ABBREVIATIONS

Ch	chitosan
CMC	carboxymethyl cellulose
HA	hyaluronic acid
HCLE	telomerase-immortalized human corneal-limbal epithelial
HPMC	hydroxypropylmethyl cellulose
IEP	isoelectric point
PAMAM	polyamidoamine dendrimers
QCM-D	quartz crystal microbalance with dissipation
RU	resonance units
SPR	surface plasmon resonance
XPS	x-ray photoelectron spectroscopy

INTRODUCTION

Ocular delivery requires the implementation of effective strategies to increase the drug residence time on the ocular surface with the aim of minimizing systemic drug side effects and reducing dosing frequency, which might improve patient compliance, one of the main lack-points of chronic

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therapies at topic ophthalmic level. In this context, the use of bioadhesive formulations able to interact with the transmembrane mucins covering the ocular surface, has gained attention in the last decades (1,2). Furthermore, some pathologies of the ocular surface, such as "dry eye syndrome", can be also beneficiated by the inclusion of bioadhesive polymers in the eye drops because of their lubricant and humectant nature (3). Recent evidences showed that transmembrane ocular mucins and their O-glycans on the cell-surface glycocalyx have important biological roles in the protection of corneal and conjunctival epithelia, such as, promoting boundary lubrication, and maintaining epithelial barrier function (4–6).

Bioadhesion of dosage forms is a complex phenomenon, involving simultaneously interfacial interactions with the living tissues and the development of adequate mechanical properties of the bulk formulation. However, whatever the nature of the materials constituting the dosage forms, remanence at the corneal surface primarily depends on the characteristics of the adhesive interface that can be created with the transmembrane ocular mucins. Thus, the development of a dedicated *in vitro* technique specifically designed to evaluate the interaction of polymers with transmembrane ocular mucins would be a valuable tool for a rational selection of excipients of ocular drug formulations. Considering the low polymer concentration used in topic ocular administration, the classic techniques based on tensiometric or rheological measurements are not very suitable for evaluating interfacial adhesive properties but rather bulk properties of the formulations, such as hydrogels. Furthermore, some rheological studies consisting in mixing mucins to the polymers to be studied for evaluating possible interactions typically require the use of relatively high amounts of mucins. It is a severe limitation in the case of ocular mucins, which are produced in very low extent, are not commercially available and must be substituted by gastrointestinal porcine mucins in most of the cases (7,8), considerably limitating the pertinence of results. Besides, these "bulk-like" *in vitro* mucoadhesion tests only give a macroscopic and indirect measurement of polymers-mucin interactions thus it is not possible to evaluate the interaction at a molecular level.

Apart of these methods, powerful techniques such as isothermal titration calorimetry, quartz crystal microbalance or surface plasmon resonance (SPR) are currently available for the evaluation of interactions at the molecular scale. Among them, SPR is a very attractive chip-based biosensor technique, allowing the direct observation of binding events and kinetics between molecular partners. Very interestingly, it has such a high sensitivity that only small amounts of samples are required (9). Although the SPR technique was developed to evaluate specific interactions (ligand-receptor or antibody-antigen), some attempts of mucoadhesion evaluation by SPR techniques have already been carried out (10,11).

The objective of the present work was to develop an *in vitro* methodology, based on biosensor chip technology, able to evaluate the interactions of ocular transmembrane mucins with ophthalmic preparations. In a first step, the mucin layer formed on the sensor chip was deeply characterized by SPR, quartz crystal microbalance with dissipation (QCM-D) and X-ray photoelectron spectroscopy (XPS). In a second step, the interaction of transmembrane mucins either with conventionaly used polymers, or with dendrimers, as new drug delivery systems currently explored in the topical ophthalmic route (8), was assessed. It might be the first *in vitro* method specifically designed to evaluate the interaction of ocular transmembrane mucins with polymers or other adhesive molecules. It is expected that it would allow the evaluation of these interactions at molecular level. This technique could help on the comprehension of the complex mechanisms involved in mucoadhesion at the ocular surface.

MATERIALS AND METHODS

Materials

Hyaluronic acid ophthalmic grade (Mw 800,000–1,200,000 g/mol), carboxymethyl cellulose medium viscosity (CMC; 400–800 cps, 2 % solution at 20 °C) and hydroxypropylmethyl cellulose (HPMC; 1390 cps, 2 % solution at 20 °C), were purchased from Abaran Materias Primas S.L. (Madrid, Spain). Chitosan (400,000 g/mol) was supplied by Fluka (Saint-Quentin Fallavier, France) and ethylenediamine 2-carbon core PAMAM dendrimers (G4 PAMAM-NH_2, G4 PAMAM-OH, G3.5 PAMAM-COOH) manufactured by Dendritech Inc., were obtained from Sigma-Aldrich (Saint-Quentin Fallavier, France). Au-coated crystals for QCM-D analysis were provided by Q-sense A B (Gothenburg, Sweden). Reactives and materials used for the SPR experiments were obtained from Biacore-GE Healthcare (Orsay, France). All chemicals were reagent grade and used as received.

METHODS

Ocular Mucin Isolation

Telomerase-immortalized human corneal-limbal epithelial (HCLE) cells were plated on T150 flasks (Costar Corning, Inc., Corning, NY) and grown in a medium optimized for proliferation of keratinocytes (keratinocyte serum-free medium; Gibco-Invitrogen Corp.; Carlesbad, CA) to achieve confluence. After reaching confluence, cells were switched to stratification medium containing DMEM/F12 (Gibco-Invitrogen Corp.) with 1 mM $CaCl_2$, 10 ng/mL EGF

(Hyclone, Logan, UT) and 10 % calf serum (Gibco-Invitrogen Corp.) for 7 days to promote stratification and optimal biosynthesis of cell surface-associated mucins. Derivatization and mucin profile of HCLE cell cultures have been previously reported (12). Mucin was purified from stratified cultures of HCLE cells as previously described (13,14). Briefly, protein from cell cultures was extracted using RIPA buffer (150 μM NaCl, 50 μM Tris, pH 8.0, 1 % NP 40, 0.5 % deoxycholate, 0.1 % SDS) plus complete Protease Inhibitor Cocktail (Roche Biochemical; Indianapolis, IN). After homogenization with a pellet pestle, the protein extract was centrifuged at 13,500 rpm for 45 min and the protein concentration of the supernatant determined using the Pierce BCA Protein Assay Kit (Thermo Scientific; Rockford, IL). High molecular weight mucins were separated by gel chromatography on a Sepharose CL-4B size exclusion column. The void volume (Vo) containing the mucin fraction was digested with RNase A and DNase I (1 mg nuclease/100 mg protein) for 3 h. at room temperature, and further purified by isopycnic density gradient centrifugation in cesium chloride. The presence of the cell surface-associated mucin MUC16 in individual fractions was assayed by agarose gel electrophoresis and western blot using the M11 antibody (Neo Markers; Fremont, CA).

Mucin Immobilization on Surface Plasmon Resonance Chip (Biacore®)

Purified ocular mucin was immobilized on the Sia Kit Au chips (Biacore®) by incubation of the surface with 50 μl of mucin aqueous solution (80 μg/ml) over night at room temperature. Afterwards, chips were thoroughly rinsed in MilliQ® water. Once the chip was prepared and inserted on the Biacore® T100 apparatus, it was again washed with MilliQ® water at 30 μl/min (12 h). Resonance units (RU) were measured before and after the immobilization step to monitor the final amount of mucin attached to the Au chip. Calculations of immobilized mucin amounts were performed on the basis of the Biacore® standard relation: 1 RU = 1 pg·mm^{-2}. It is relevant to mention that this relation is valid for proteins in general, so in the case of highly glycosylated proteins, such as mucins, this value might not be completely accurate. The optical mucin layer was calculated considering that 1 RU correspond to a resonance angle shift of 10^{-4}° (9).

Mucin Layer Characterization

Quartz Crystal Microbalance with Dissipation Measurement (QCM-D)

Experiments were performed at 35.0±0.1 °C using a QCM-D E1, Q-sense AB (Gothenburg, Sweden). The device has been described in detail elsewhere (15). Briefly, AT cut quartz crystals oscillate at the resonant frequency (5 MHz) or at one of its overtones (3rd, 5th, 7th, 9th, 11th and 13th). The drive circuit is then open-circuited and the exponential decay of the oscillation amplitude is monitored. The dissipation, D, is defined as the fraction of energy of the oscillation that is dissipated during one period of oscillation. The resonant frequency decreases when materials are adsorbed on the crystal surface. The frequency shift (Δf) is related to the amount while the dissipation shift (ΔD) reflects the viscoelastic properties of the adlayer. An estimation of the adsorbed mass can be made according to the Sauerbrey equation (16) when the adlayer is homogeneous, not slipping and rigid, or according to the Voigt model when the adlayers present viscoelastic behavior (17). Ocular mucin immobilization on the gold sensor was achieved by circulating a 80 μg/ml aqueous solution of ocular mucin at 60 μl/min over the sensor's surface until stabilization, using a peristaltic pump (Ismatec IPC-N4). After rising with milliQ® water at the same flux, the running buffer was changed to PBS pH 7.4 until stabilization. Both oscillation frequency shifts and energy dissipation changes were monitored all over the assay. Furthermore, all frequency shifts were normalized with the overtone number.

X-Ray Photoelectron Spectroscopy (XPS)

The mucin layer formed on the Biacore® Au chip was evaluated by XPS analysis. High-resolution XPS data were acquired with a Thermo VG Scientific ESCALAB 250 spectrometer with a hemispherical electron analyser using a monochromatic Al Kα X-ray source operating in an ultra high vacuum chamber with a base pressure in the 5×10^{-10} mbar range. The spectra were recorded at normal emission take-off angle, using an energy step of 1 and 0.1 eV and a pass-energy of 100 and 40 eV for survey spectra and core levels respectively. Compositional analysis by XPS were performed by assuming a homogeneous distribution of atoms present on the gold surface covered by the mucin layer and using tabulated atomic sensitivity factors.

From the attenuation intensity of the gold Au4f peak, one can estimate the thickness (d) using the exponential attenuation:

$$I_s = I_S^0 \cdot e^{\left(\frac{-d}{\lambda \cos\theta}\right)}$$

Where Is^o is Au4f intensity before coating, Is the Au4f intensity after coating, λ is the mean free path of Au4f electrons in the mucin layer, and θ is the photoelectron emission angle (relative to the normal surface).

Screening of Adhesion Behaviour of Polymers and Dendrimers with Ocular Transmembrane Mucins by Surface Plasmon Resonance

Polymers and Dendrimers Preparation

Three polymers typically included in ocular topical formulations, hyaluronic acid (HA), carboxymethyl cellulose (CMC), and hydroxypropylmethyl cellulose (HPMC) were chosen at the concentrations generally used for ocular surface administration in clinical practise (0.2 % HA, 0.5 % CMC, 0.3 % HPMC) (18). A chitosan solution (Ch) was also evaluated. Although there is no commercial ocular formulation including chitosan yet, it has been extensively studied as a mucoadhesive agent for the improvement of drug ocular bioavailability (19). Polymer solutions, except chitosan, were prepared by stirring at room temperature in PBS, pH 7.4 overnight and filtering (0.45 μm). In the case of Ch, the commercial polymer was first depolymerised in order to reduce its molecular weight to 20,000 g/mol (20). Once prepared, depolymerised chitosan was dissolved in acetic acid solution (pH 6) with a final polymer concentration of 1 % and filtered. In addition, PAMAM dendrimers, new drug delivery systems currently intended for ophthalmic administration, were also evaluated. The G4 dendrimers with amino and hydroxyl surface groups and the G3.5 PAMAM dendrimer with carboxylic groups at the surface were prepared at a concentration of 2 % in PBS, pH 7.4, according to other authors (8). Briefly, dendrimers in the methanolic commercial PAMAM solution were evaporated under vacuum (Rotavapor R-124 Büchi. Switzerland) and subsequently dissolved in PBS pH 7.4.

Surface Plasmon Resonance Binding Experiments

Solutions of linear polymers and dendrimers were flowed over the mucin coated sensor chip surface within the Biacore® T100 apparatus for 300 s at 10 μl/min. PBS, pH 7.4, was used as running medium except for Ch experiments, where acetic acid solution (pH 6) was used. Afterwards, the sensorgram (RU *versus* time) was collected until equilibrium was reached. All studies were performed in triplicate at the ocular surface physiological temperature: 35 °C.

Ocular Mucin-PAMAM Dendrimers Surface Plasmon Resonance Isotherms

Five concentrations of PAMAM-NH_2 and PAMAM-OH dendrimer in PBS (pH 7.4) or in acetate buffer (pH 5.5) were used in SPR assays. All analysis were performed at 35 °C, with a 600 s period of ligand-analyte association and 600 s period of dissociation, at 10 μl/min. Dendrimer concentrations (10–80 nM) and contact time were optimized in order to approach the steady state at all concentrations. The corresponding isotherms were prepared by plotting the amount of dendrimers attached to the ocular transmembrane mucin layer *versus* dendrimer concentration.

Chip Regeneration

After SPR assays, the Au chips were carefully separated from their plastic holders by addition of ethanol and the gold surfaces were cleaned in a 5:1:1 mixture of MilliQ® water, NH_3 (25 %) and H_2O_2 (30 %), respectively, before heating for 15 min at 70 °C. Afterwards, they were rinsed with water and dried with N_2. Subsequently, chips were exposed three times, 15 min each, in a UV-O_3 chamber (UV/ozone procleaner™ Bioforce nanosciences. Paris, France) (21). Samples were rinsed with ethanol and dried with N_2 before each exposition. XPS evaluation of the regenerated gold surface was performed to determine the absence of mucins on the chip surface. The same procedure was followed to prepare the gold-coated crystals prior to QCM-D studies.

Statistical Analysis

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

RESULTS

Ocular Mucin Isolation

Ocular surface mucins were purified from stratified cultures of human corneal limbal epithelial (HCLE) cells using size-exclusion chromatography followed by isopycnic density gradient centrifugation (Fig. 1). HCLE cells produce cell surface-associated mucins which can be distinguished on the basis of their molecular size as they are eluted as a single peak in the void volume (Vo, Fig. 1a). As determined by western blot using the M11 antibody against MUC16, gradient centrifugation produced aliquots of purified mucin with a buoyant density range of 1.26 to 1.44 g/ml (Fig. 1b). Soluble mucins in fractions within this density range were combined and used for further study.

Mucin Layer Preparation and Characterization

The first step in the development of a new mucoadhesion *in vitro* test based on SPR measurements is characterization of

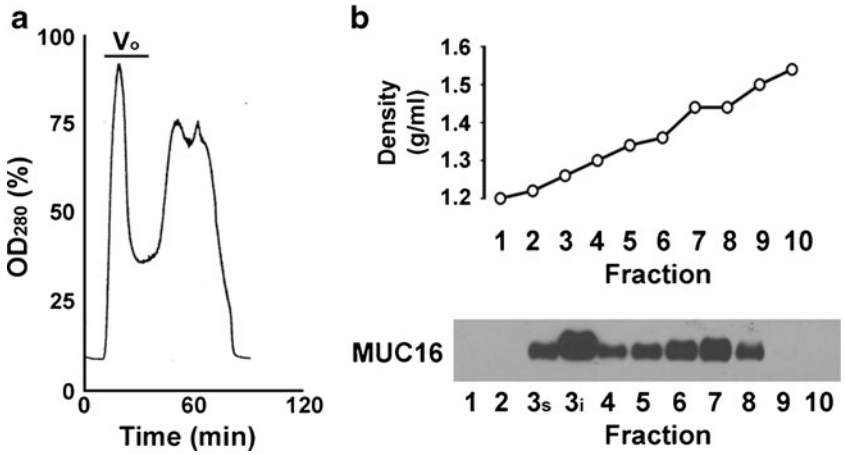


Fig. 1 Purification of ocular mucin. (**a**) Sepharose CL-4B chromatography of cell lysates from stratified HCLE cell cultures. The column eluate was monitored for absorbance at 280 nm. The void volume fraction (Vo) containing the high molecular weight mucins was collected and further purified by gradient centrifugation. (**b**) Density gradient centrifugation of the Vo fraction in cesium chloride. One-ml aliquots were electrophoresed on a 1 % (w/v) agarose gel and then Western blotted onto nitrocellulose as described in Materials and Methods. The blot was probed with MUC16-mucin specific M11 antibody. Fraction 3 contained both soluble (s) and insoluble (i) mucin. Soluble fractions 3 to 8 were combined and used for further study.

the ligand (mucin) layer on the gold chip. In this purpose, QCM-D, SPR and XPS assays were performed.

Quartz Crystal Microbalance with Dissipation Measurement (QCM-D)

According to Fig. 2b, in milliQ® water, mucins progressively adsorb on a gold surface, resulting in a decrease in frequency of vibration of the quartz crystal until stabilization. The maximal amount of hydrated mass adsorbed was 2.35 ng/mm² calculated according to the Sauerbrey equation. The dissipation factor (D) provides a measure of energy loss in the system and contains information about film interactions with the bulk solution. Generally, rigid structures have minimal effect on the dissipation, whereas thick or flexible structures increase the dissipation so it can be seen as a measure of the rigidity or viscoelasticity of the adsorbed film (22). The observed normalized frequency shift at the various overtone overlaps indicated the presence of a rigid layer with dissipation lower than 0.5. However, an additional decrease in frequency was recorded when running media was changed from deionised water to PBS, pH 7.4, (Δf comprised between −7 Hz and −10 Hz and a ΔD of 2 to 3.5) showing a relatively more extended conformation of the protein in presence of ions (30,31).

Surface Plasmon Resonance (SPR)

The evaluation of the mucin layer was also assessed by surface plasmon resonance. Biacore® Au chips were prepared by deposition of 50 µl of an aqueous ocular mucin solution (0.08 mg/ml). After an overnight incubation, chips were mounted on their plastic containers and measured in the Biacore® apparatus, obtaining a mean RU increment of 4,954±204. A critical parameter in SPR studies is the surface density of the ligand (24). Therefore, the mucin deposition data on the four channels of each chip were statistically evaluated (one-way ANOVA analysis), showing statistically similar values of ΔRU ($p>0.05$), that is mucin deposition, in all channels and chips used. The amount of mucin immobilized on the Au surface was in the same order as those obtained from covalent mucin immobilization on a CM5 chip (dextran modified Au surfaces) (25). According to the Biacore® protein standard relation (1RU = 1 pg/mm^2), an immobilization of 4.9±0.2 ng/mm^2 of ocular mucin can be assumed. Comparing this value with those obtained for mass adsorption on gold surface after D-QCM experiments surface saturation was presumed. However, to confirm that all mucin chains present on the chip surface were covalently attached to the chip surface, extensive washes of the immobilized chip surface with vehicle were performed for hours in the Biacore® system, with no change on the RU values (data non shown). All these studies helped us to assume the saturation of the gold chip by the mucin layer. The optical thickness of this mucin layer determined by the shift in the resonance angle of the layer was estimated to be 2.62±0.01 nm.

X-Ray Photoelectron Spectroscopy (XPS)

The so-performed quantitative determination of the mucin layer was additionally completed by X-ray photoelectron spectroscopy (XPS) analysis. XPS scans were used for the semiquantification of atoms present on the gold surface covered by the mucin layer (26). According to Fig. 2b, the survey spectrum showed an attenuation of gold (see the decrease of the peak area for Au4f between 92 and 80 eV). N1s was clearly detected from the survey scan in the mucin layer and

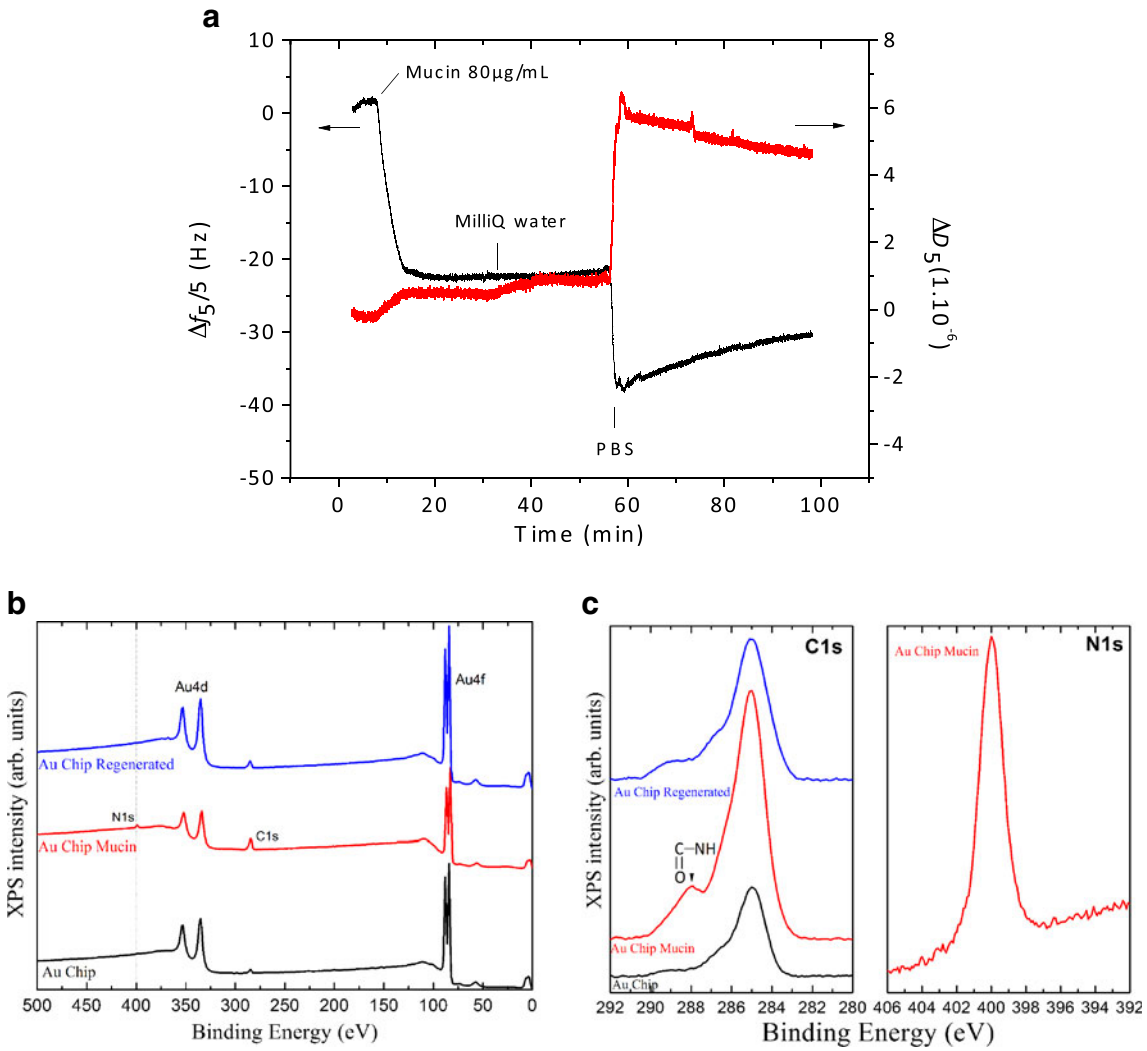


Fig. 2 Characterization of transmembrane ocular mucin layer (**a**) QCM-D Normalized frequency (*black*) and dissipation (*red*) shifts recorded at the 5th overtone during adsorption of mucin on gold-coated surface and rinsing with MilliQ® water and PBS buffer. (**b**) XPS survey spectra of Au chip (*black*), ocular mucin-coated Au chip (*red*) and after surface cleaning of mucin coated Au chip (*blue*) in a 5:1:1 mixture of MiliQ water, NH_3 (25 %) and H_2O_2 (30 %) 15 min at 70 °C + UV-O_3 chamber. The energy positions of nitrogen, carbon and gold are also indicated by *vertical lines* in the figure. The attenuation of gold intensity and the presence of N 1 s peak in mucin coated Au chip suggest the formation of a thin layer of mucin on Au chip. (**c**) High-resolution XPS spectra of C 1 s and N 1 s core levels are also shown to asses the presence of peptidic linkages -NH-C = O, (indicated by *vertical line* at 288 eV) typical of proteins.

a second maximum was detected at 288 eV corresponding to carbon atoms from the peptidic linkage -NH-C=O, showing the presence of mucins on the gold surface (Fig. 2c). The thickness of the mucin layer determined by XPS was around 1 nm. This data correlates well with the previous QCM-D and SPR studies, taking into account that these measurements are performed in dry conditions, so the highly hydrated and glycosylated portions of the mucins might be deeply modified.

Adhesion Behaviour of Polymers and Dendrimers with Ocular Transmembrane Mucins by Surface Plasmon Resonance

Initially in a SPR experiment, the sensor chamber is filled with running buffer. When analytes (polymer or dendrimer molecules) bind to ligands, they replace buffer solution molecules from the surface, which can be optically detected as an increase of the RU response, until reaching a plateau. Subsequently, when the analyte dissociates from its ligand, the running solution replaces the analyte solution over the mucin-coated surface and a partial decrease in the RU signal is detected. The total increase in RU after this washing step ("stability RU") is related to the amount of analyte retained on the ligand layer (9). The profiles obtained in evaluation of mucin-polymers interactions (Fig. 3) showed the typical profile of non-specific interactions, with an initial rapid uptake, followed by a slower process. Other authors have attributed this second step to interface reorganization (11). According to SPR results, only chitosan demonstrated statistically significant permanent chemical interactions with transmembrane ocular

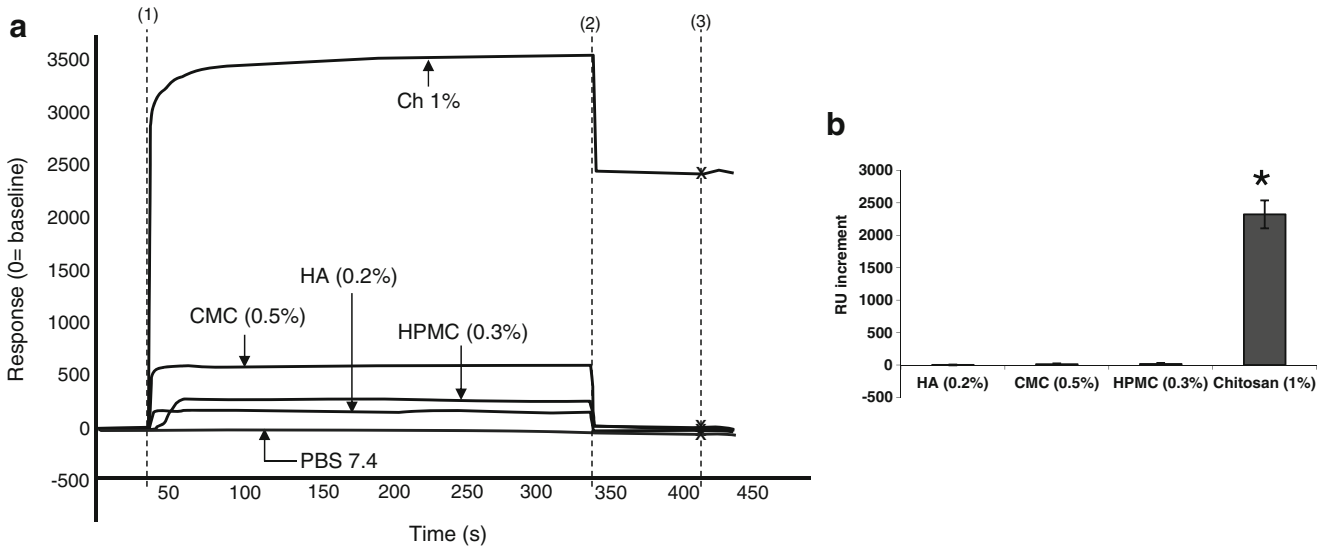


Fig. 3 (**a**) SPR sensorgrams of the interaction of polymers in PBS pH 7.4 -or acetic acid solution pH 6 in the case of Chitosan- (analyte) with transmembrane ocular mucins (ligand). Runs were performed at 35 °C. (1) sample injection; (2) washing step: (3) stability. (**b**) RU increment observed after rinsing (stability step), indicative of polymer permanent interaction on the mucin surface. *Asterisk* represents statistic differences in RU stability values ($p < 0.05$). $n=3$.

mucin surface. While HA, CMC and HPMC were unable to be retained on the mucin layer.

The SPR mucoadhesion method developed was used to test the interaction of transmembrane ocular mucins with PAMAM dendrimers with three different chemical groups on the surface: PAMAM-NH_2 (64 amino groups), PAMAM-OH (64 hydroxyl groups) and PAMAM-COOH (64 carboxylic groups). Interestingly, according to Fig. 4, all evaluated PAMAM dendrimers showed remarkable interactions with the membrane-associated ocular mucin layer. PAMAM-NH_2 and PAMAM-OH showed statistically similar values of ΔRU. Although PAMAM-COOH also adhered to the transmembrane ocular mucin layer, the intensity was lower and data showed less reproducibility (high standard deviation in the ΔRU). Additionally an anomalous non-saturating profile observed in the sensorgram (Fig. 4). Calculations were performed to achieve theoretical approximations to determine the number of dendrimer layers on the apparent mucin layer surface. Values of 1.26, 1.07 and 0.59 layers were obtained for -NH_2, -OH and -COOH PAMAM dendrimers.

Additional experiments were performed with PAMAM-NH_2 and PAMAM-OH to further analyse their interactions

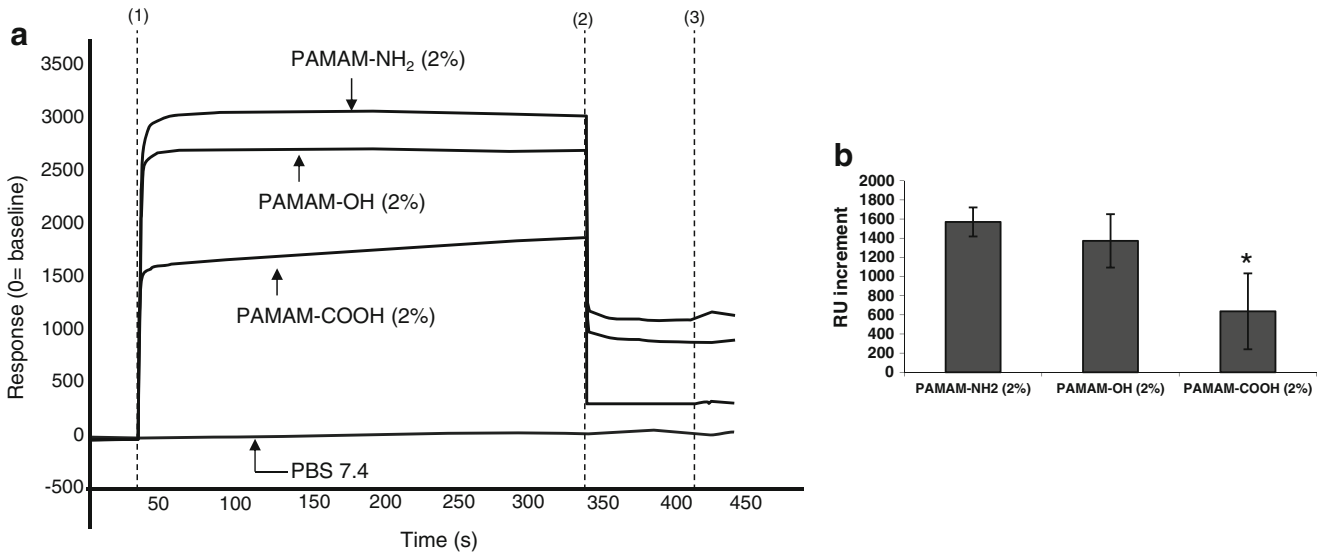


Fig. 4 (**a**) SPR sensorgrams of the interaction of dendrimers at 2 % in PBS pH 7.4 (analyte) with transmembrane ocular mucins (ligand). Runs were performed at 35 °C. (1) sample injection; (2) washing step: (3) stability. (**b**) RU increment observed after rinsing (stability step), indicative of dendrimer permanent interaction on the mucin surface. *Asterisk* represents statistic differences in RU stability values ($p < 0.05$). $n=3$.

with transmembrane ocular mucins. Different concentrations below the saturation of the apparent mucin layer surface (monolayer formation) were put in contact with the mucin layer and the interaction was evaluated by SPR. The RU increment obtained after equilibrium for each concentration was used to calculate the amount of dendrimers theoretically attached per apparent surface. Isotherms were then built by plotting those values against dendrimer concentrations (Fig. 5). Linear correlations were observed, considering the slope as indicative of the dendrimers association tendency to the ocular mucin layer: PAMAM-NH_2: 5.86 mg/mm^{2}μM; PAMAM-OH: 5.23 mg/mm^{2}μM. Similar values were observed for both dendrimers. Further experiments were repeated at low pH: 5.5 (Fig. 5), a pathological value of tear pH, typical of some diseases. At this condition, the primary amino groups of PAMAM-NH_2 remain protonated and the tertiary amines present in the inner structure of both PAMAM dendrimers are partially charged (37), meanwhile the mucin pH might not sensible change (IEP = 2–3). As can be observed, this increment in the net positive charge of PAMAM-NH_2 led to an increase in association tendency (7.84 mg/mm^{2}μM), while PAMAM-OH was not affected (4.66 mg/mm^{2}μM).

DISCUSSION

Ocular Transmembrane Mucin Layer

In this study, human membrane-associated corneal mucins obtained from stratified corneal cultures were used. In accordance with literature, mucins in solution adopt a compact tertiary structure at pH 7 (milliQ® water) in which the hydrophobic peptide residues are exposed only as they encounter a hydrophobic surface (11) such as the gold chip surface, which would bind exclusively to unglycosylated protein domains (27). This binding can occur through strong covalent bonds between gold atoms and thiol groups (Cys residues) present in adsorbing molecules (30). The created mucin layer might result in a mucin glycoproteins extended conformation with loops and tails, predisposed to interchain interactions (27). This extended conformation might artificially reduce the thickness of the mucin layer, from a dense thick cell surface glycocalyx that extends 200–500 nm from the plasma membrane *in vivo* (4), to a few nanometers as was observed by SPR, QCM-D and XPS studies. In addition, the relatively low viscoelasticity observed for the mucin layer after QCM-D studies might be also explained by these multiple interactions of non-glycosylated mucin domains with the gold surface, which would limit the chain's movement. Therefore, it is possible that the mucin layer formed on the gold chip might have the orientation suggested in Scheme 1. At these conditions, a reduction of free chain movements might be expected.

Mucoadhesion Studies by Surface Plasmon Resonance

Mucoadhesion is a complex process and numerous theories have been presented to explain the mechanism(s) involved, describing mechanical-interlocking, electrostatic, diffusion-interpenetration, adsorption, and surface fracture processes, *etc.* (28,29). It is widely accepted that mucoadhesion of various formulations, results from the combination of two phenomena; (i) the development of interfacial interactions with living tissues, which may involve physical interpenetration between the mucoadhesive polymer and the mucin chains, and which is followed by non-covalent bonding due to hydrogen bond formation, electrostatic interactions or others depending on the nature of the adhesive material (28,30) (ii) the mechanical resistance of the bulk adhering materials (bulk formulation

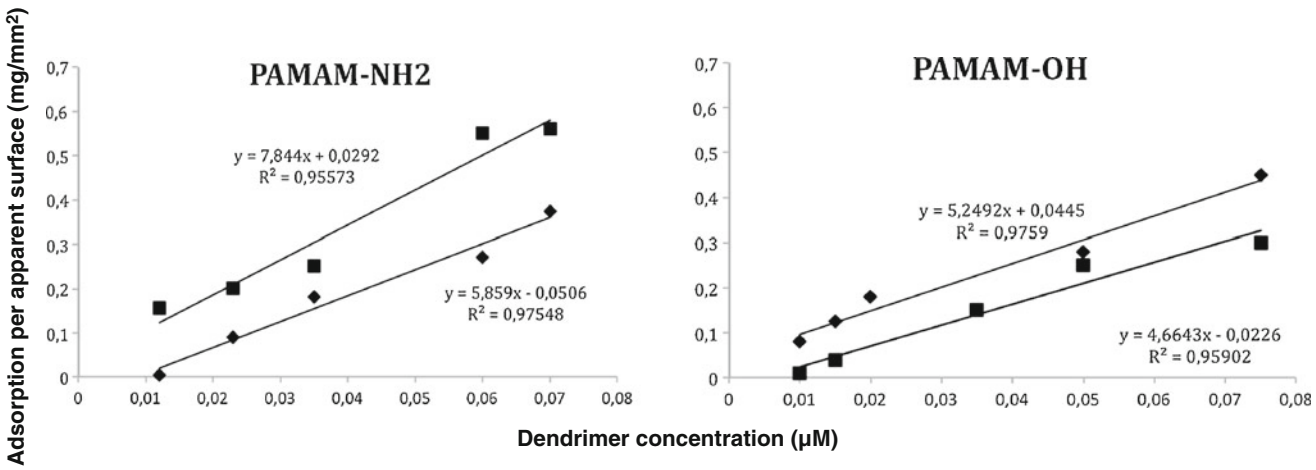
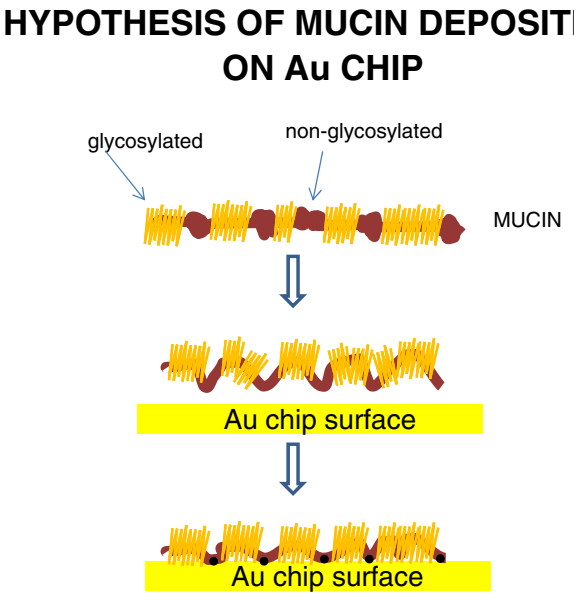


Fig. 5 Isotherms of ocular mucin-dendrimer association. Amount of dendrimers attached to the transmembrane mucin layer per apparent surface G4 PAMAM-NH_2 and G4 PAMAM-OH at pH 7.4 (PBS) *diamonds* and at pH 5.5 (acetate buffer) *squares*. The slope is indicative of the attachment tendency.



Scheme I Hypothesis of transmembrane ocular mucin chains immobilization on gold surface. Hydrophobic peptide residues of the unglycosylated domains of mucin are exposed in the proximity of the gold chip surface, developing strong covalent bonds. The created layer results in a mucin glycoproteins extended conformation with a thickness of a few nanometers.

and tissues). The SPR technique has been chosen for probing the interfacial interaction phenomena between ocular mucins and adhesive molecules, because they are the basis for the development of strongly bioadhesive formulations. As previously suggested, the configuration of the mucins at the surface of the chips corresponded probably to the presence of an almost monomolecular layer of glycoproteins, making this surface suitable for the observation of secondary bonding, but more unlikely chain interpenetration phenomena leading to mechanical interlocking or expanded secondary bonding. Indeed, chains interdiffusion is a time-dependent diffusion phenomenon (31) where a minimum thickness of both parts (mucin and polymer) is needed (32), The penetration/diffusion/entanglement processes between polymers and mucin chains can enhance "interfacial" interactions, but also can be more easily investigated through "bulk-like" mucoadhesion *in vitro* tests (tensiometric measurements, rheological studies, *etc.*), where both mechanisms are inseparable. This was favourable, as the SPR method should rather allow the determination of chemical interactions between rather well defined surfaces, therefore, can be useful to discern between the mucin-polymer chemical interactions at molecular scale.

Very well known hydrophilic polymers, especially electrically charged polymers, rich in OH, COOH and NH_2 groups demonstrated a high mucoadhesive capacity (33). The ocular mucin-polymer interactions of two negatively charged polymers (HA and CMC) and one positively charged polymer (chitosan) were evaluated. Additionally, a non-charged polymer (HPMC) was also tested. In all cases, solutions of these polymers at the concentrations typically used for ocular administration (and not hydrogels) were employed. Chitosan is only soluble at pH lower than 6.5, even when the molecular weight is reduced, which is the case of the one used in the present work, that is why a acetic solution (pH 6) was used instead of PBS pH 7.4. Both pH (6 and 7.4) are accepted at the ocular surface. Furthermore, considering that the isoelectric point (IEP) of mucins is around 2–3, no significant changes can be expected in the mucin layer at these conditions (34).

According to results, only chitosan developed statistically significant permanent chemical adherence to the mucin layer. This behaviour could explain the promising results observed for chitosan-based drug delivery when administered on the ocular surface (19). Chitosan, a polycation (pKa = 6.2), has been reported to bind mucin, a polyanion (pKa = 2.6), *via* ionic interaction between primary amino groups and the sialic and sulphonic acid substructures of glycosylated chains of mucins. Additionally, the hydroxyl and amino groups of chitosan may also interact with mucin *via* hydrogen bonds (28). On the contrary, it may suggest that a prior chain interdiffusion is mandatory for HPMC, HA and CMC polymers to promote the formation of a sufficiently stable adhesive interface. In the case of HPMC, its ability to increase the drug retention time after ocular administration has been claimed to be related to its viscosity enhancing capacity (1), more than any intrinsic mucoadhesive nature, showing "negative interaction" with mucin after rheological studies (35). For both anionic polymers, HA and CMC, although it was claimed that they are able to develop strong hydrogen bonding with mucin molecules (1,36), it seems that these interactions need to be accompanied by chains interpenetration. In fact, it was already demonstrated that changes in pH solutions of HA did not influence the extension of its interaction with mucins. Thus, its adhesion behaviour might be mainly governed by physical chain interpenetration, more than chemical interaction with mucin chains (1).

Among the different new drug delivery systems currently under study, dendrimers, more precisely polyamidoamine dendrimers (PAMAM), have gained increasing attention. These new systems have potential medical applications due to the combination of unique chemical properties with high biocompatibility, low immunogenicity, and ease of synthesis on a large scale with reasonable manufacturing cost (37). At the ocular level, some studies have demonstrated their utility in the increase of drug bioavailability after topical administration in solutions (8) and as hydrogels based on PAMAM and polyethylene glycol (PEG) (37). One of the most important characteristics of dendrimers is the high density of active chemical groups located on their surface. In comparison to linear polymers, these multivalent systems might promote a co-operative effect leading to a large increase in

reactivity (38). Effectively, in contrast to linear polymers, the three different PAMAM dendrimers evaluated developed adhesion to the transmembrane ocular mucin surface whatever their chemical surface group (NH_2; OH; COOH). In an interesting work, Griffiths *et al.* (39) evaluated the interaction of PAMAM-NH_2 dendrimers (generation 2.0 and 4.0) with porcine gastric mucin (type III) in solution, by pulsed-gradient spin-echo NMR and small-angle neutron scattering. They also observed that these dendrimers (concentration 0.5 % wt) experienced significant interaction with mucins at pH 7. At neutral pH all primary amino groups of PAMAM-NH_2 dendrimers are protonated (40), which should promote electrostatic interactions with the anionic sialic groups of mucins. According to our results, the PAMAM-mucin interactions observed were statistically similar for PAMAM-NH_2 and PAMAM-OH, although the latter can only develop weaker non-ionic interactions, such as hydrogen bonds, with mucins. This behaviour would suggest a remarkable contribution of non-ionic interaction in the development of ocular mucin-dendrimer interactions. In fact, other authors have previously suggested that polymers exhibiting high density of available hydrogen bonding groups would be able to intensely interact with mucins (28). Additionally, the pH-dependent behaviour of PAMAM-NH_2 suggests that at these pH conditions, this dendrimer might be able to perform even stronger interaction with ocular mucins, which could be of relevance in the development of dendrimer based drug delivery systems for the treatment of ocular surface pathologies involving reduction of tear pH, such as ocular inflammation.

Interestingly, PAMAM-COOH dendrimers also developed adhesion on the mucin layer, in spite of the electrostatic repulsion with the negatively charged mucin surface. Other authors also observed interaction between PAMAM-COOH (generation 3.5 and 5.5) and gastric mucins at pH 7, due to the formation of hydrogen bonds between charged carboxylic groups of the dendrimer and the sugar residue on the mucins side chains (39). Furthermore, the non-glycosylated domains of mucins posses positively charged amino acid residues that could establish electrostatic interactions with negatively charged groups of the polymers, as long as the repulsive forces between negative charges (polymer and O-glycan chain) are efficiently screened by salt (23). This scenario could explain the more erratic behaviour observed for the interaction of PAMAM-COOH with ocular mucins in this work.

CONCLUSIONS

In this work, we have explored some of the many possibilities of SPR biosensors in the *in vitro* evaluation of mucoadhesion, offering the first *in vitro* method specifically designed to evaluate the interactions of ocular transmembrane mucins with polymers or other macromolecules. The presented technique allows the evaluation of interfacial mucins-polymer interactions at the molecular level, which, in combination with "bulk-like" macroscopic studies, is a useful tool to give a better understanding of the complex mechanisms of mucoadhesion on the ocular surface. In this context, according to our findings, it can be concluded that, among the linear polymers evaluated, only the cationic linear chitosan was able to chemically interact with the transmembrane ocular mucin surface. In addition, the mucoadhesion test developed in this work was used to evaluate the ocular mucoadhesion of one of the newest and more promising drug delivery systems currently under study: dendrimers. Results demonstrate that PAMAM dendrimers can develop permanent chemical interactions with transmembrane ocular mucins, especially PAMAM-NH_2 and PAMAM-OH, at physiological and pathological tear pH. Furthermore, the carboxylic terminal PAMAM dendrimer was also able to perform interaction with such a mucins to a remarkable extent. These results reinforce the idea of the high potential that dendritic structures could have on the development of new drug delivery systems able to increase the residence time of drugs on the ocular surface, thanks to their interaction with transmembrane ocular mucins. Appendix

ACKNOWLEDGMENTS & DISCLOSURES

Dr. Bravo-Osuna would like to thank the Institute de Chimie du Centre National de Recherche Scientifique du France (CNRS) for financial support. She is also very thankful to Mrs. S. Mazzaferro (CNRS UMR 8612, Université Paris Sud) for the preparation of low molecular weight chitosan, to Dr. V. Andrés and Dr. M. Vicario (University Complutense of Madrid) for their useful comments, and to Dr. J.A. García (Surface Physics and Engineering Department–CSIC) for kindly help in XPS discussion. Dr. Bravo-Osuna, Dr. Herrero-Vanrell and Dr. Molina-Martínez would like to thank to Research Group UCM 920415 (GR35/10-A) and MAT2010-18242 for financial support. Dr. Argüeso would like to thank NIH/NEI Grant No. R01EY014847 (PA) for financial support. Authors would like to thank the IOTDYS (Université Paris VII) for XPS analysis.

APPENDIX

Chip Regeneration

Some authors have already observed the strong interaction established between mucins and polymers during SPR experiments, which makes difficult, even impossible to separate both ligand and analyte without the risk of altering mucin structure (10,11). Several detergents as well as acidic

and basic solutions have been proposed to regenerate ligand surfaces in SPR studies (41), however, all recommended solutions were useless in the regeneration of a ligand layer in the present experimental work (data non shown). In order to reuse chips, it was necessary to develop a total regeneration method, which removed not only analyte but also ligand from the gold surface. The method selected, known as "basic piranha", was able to eliminate all bound protein. This method, in combination with surface exposure to a UV-ozone chamber, was used to recondition the Au surface. XPS studies were performed on regenerated Au surfaces. According to Fig. 2b, c, the intensity of Au4f reached levels similar to the non-treated gold surfaces. Additionally in the core levels, neither N1s peak nor second maximum at 288 eV were recorded in the regenerated Au chip scan, indicative of the absence of proteins. Further, the cleaning procedure does not alter the chemical state since there is not energy displacement of peak positions.

Additionally, the baselines of "recycled" chips were monitored on Biacore® showing statistically similar ($p > 0.05$) values before and after regeneration.

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