A water-soluble, mucoadhesive quaternary ammonium chitosan-methyl-β-cyclodextrin conjugate forming inclusion complexes with dexamethasone.

Anna Maria Piras[1,*], Ylenia Zambito[1], Susi Burgalassi[1], Daniela Monti[1], Silvia Tampucci[1], Eleonora Terreni[1], Angela Fabiano[1], Federica Balzano[2], Gloria Uccello-Barretta[2], Patrizia Chetoni[1]

[1]Department of Pharmacy, University of Pisa, via Bonanno 33, 56126 Pisa, Italy

[2]Department of Chemistry and Industrial Chemistry, University of Pisa, via Moruzzi 13, 56124 Pisa, Italy

*Corresponding author: Anna Maria Piras, Department of Pharmacy, University of Pisa (Pisa, Italy); e-mail: anna.piras@unipi.it; Tel: +39-050-2219704

Abstract

The ocular bioavailability of lipophilic drugs, such as dexamethasone, depends on both drug water solubility and mucoadhesion/permeation. Cyclodextrins and chitosan are frequently employed to either improve drug solubility or prolong drug contact onto mucosae, respectively. Although the covalent conjugation of cyclodextrin and chitosan brings to mucoadhesive drug complexes, their water solubility is restricted to acidic pHs. This paper describes a straightforward grafting of methyl-β-cyclodextrin (MCD) on quaternary ammonium chitosan (QA-Ch60), mediated by hexamethylene diisocyanate. The resulting product is a water-soluble chitosan derivative, having a 10-atom long spacer between the quaternized chitosan and the cyclodextrin. The derivative is capable of complexing the model drug dexamethasone and stable complexes were also observed for the lyophilized products. Furthermore, the conjugate preserves the mucoadhesive properties typical of quaternized chitosan and its safety as solubilizing excipient for ophthalmic applications was preliminary assessed by *in vitro* cytotoxicity evaluations. Taken as a whole, the observed features appear promising for future processing of the developed product into 3D solid forms, such as controlled drug delivery systems, films or drug eluting medical devices.

Keywords.

Chitosan, Cyclodextrin, Inclusion Complex, Mucoadhesion, conjugated polymers

1. Introduction

The absorption of a drug consists of two processes in series, namely drug dissolution followed by drug permeation across biological barriers. It is known that a kinetic process consisting of two stages in series is controlled by the more resisted one and with drugs poorly soluble in physiological fluids, the dissolution may be the rate-determining stage. In these cases, there may be a risk of too low a bioavailability. Hence, the drug dissolution rate should be increased to enhance drug bioavailability. One of the strategies used to increase the dissolution rate of hydrophobic drugs is to prepare drug inclusion complexes with cyclodextrins (CD). Cyclodextrins are a family of compounds made up of 6-8 α-D-glucopyranoside units bound together in a ring by 1-4 bonds. They have been used widely, thanks to their ability to strongly interact with a wide variety of hydrophobic compounds via host-guest inclusion complexation [1, 2]. In recent years, several studies have been carried out to design CD grafted polysaccharides, in view of obtaining highly versatile materials maintaining the properties of both CD and polysaccharides [3]. In particular, the CD conjugation to mucoadhesive polysaccharides is aimed at combining the solubility enhancement of lipophilic drugs with a prolonged drug contact with the absorption site. This later aspect is especially significant for ocular, oral, buccal, nasal, vaginal, and rectal dosage forms. Among the lipophilic drugs, dexamethasone (DEX, LogP=1.83[4]) has commonly been investigated. When administered as eye drops, DEX bioavailability is mostly confined to the anterior segment of the eye and a renewed interest, in transforming the classical drug DEX into a new blockbuster medicine, is presently attracting scientists as well as commercial competitors[5]. For this purpose, several formulations have been reported for the enhancement of DEX bioavailability, including microparticles, nanoparticles, liposomes and aqueous drops containing CDs[6].

In the formulation of eye drops, the optimal polymer to be used as additive should be mucoadhesive, without increasing the viscosity of the solution to an excessive extent[7]. Chitosan derivatives are effective to this end[8]. Chitosan (CS) (1,4-2-amino-2-deoxy-β-D-glucan) is a non-toxic, biocompatible and biodegradable polymer obtained by deacetylation of chitin, which is one of the most abundant polysaccharides in nature. CS has widely been used for pharmaceutical and biomedical application [9, 10], thanks also to its antimicrobial [11] and mucoadhesive features [12, 13]. Additionally, it is possible to play with CS functionalities and investigate on CS derivatives displaying additional features, e.g. antioxidative [14]. Among CS derivatives, chitosan-cyclodextrin (CS-CD) conjugates were prepared showing promise of increasing drug bioavailability [15], yet their poor solubility in the neutral or alkaline environment of the physiological fluids was found to limit their pharmaceutical applications. Therefore, the preparation of a soluble macromolecular CS-CD conjugate has been carried out [16]. Such a product was obtained via quaternization of the pre-formed CS-CD conjugate, a reaction that could result in a decreased cyclodextrin ability to form inclusion complexes with drugs. Indeed, guest inclusion in CD generally occurs via the wider toroid opening, where secondary hydroxide groups are found. Hence, the steric hindrance effects due to the substitution of the hydroxide moieties may cause an important decrease of the association constant of the relevant complexes[17].

On this basis, in the present study a water-soluble CS derivative, quaternized beforehand and further functionalized with CD through a long arm spacer, has been used as a complexing agent to improve DEX solubility. This approach is expected to rule out any non-specific reaction that could generate quaternized CD and thus limiting its complexing properties. The pre-synthesized quaternary ammonium-chitosan derivative, i.e. *N,O*-[*N,N*-diethylaminomethyl(diethyldimethylene ammonium)$_n$ methyl]chitosan (QA-Ch60), is soluble irrespective of pH and it is known to have mucoadhesive properties[18]. QA-Ch60 was shown to bear short pendant chains, each

containing a small number of adjacent quaternary ammonium groups, partially substituted on the polymer repeating units [19, 20].

Additionally, a highly soluble (20% in water at 20°C) methyl-β-cyclodextrin (MCD) was used, selectively substituted on O-2 positions and carrying reactive primary OH on C6, available for polymer grafting. The straightforward grafting of MCD on QA-Ch60 was mediated by hexamethylene diisocyanate (HMDI), leading to a conjugate product (QA-Ch-MCD) with a 10- atom long spacer.

2. Materials and Methods

2.1. Materials

CS (weight-average molecular weight Mw 50-190 kDa and deacetylation degree (DD) 75-85%), 2-diethylaminoethyl chloride (DEAE-Cl) hydrochloride, dimethyl sulfoxide (DMSO), 1, 6 -hexamethylene diisocyanate (HMDI), and triethylamine (TEA) were from Sigma-Aldrich (St. Louis, MO 63103 USA). Dexamethasone (DEX) from Merck Sharp & Dohme research lab, methyl β-cyclodextrin (MCD, KLEPTOSE® CRYSMEB EXP) from Roquette (LESTREM – FRANCE), standard RC Dialysis membranes (MWCO 12500) from Spectra/Por® (CA, USA). HMDI was distilled under reduced pressure (65 °C/0.2 mbar); DMSO was refluxed over calcium hydride and distilled at reduced pressure (24°C/0.2 mbar); TEA was refluxed over potassium hydroxide and distilled before use. Hog Gastric Mucin (HGM) was from Carl Roth GmbH & Co KG, Karlsruhe, Germany. All other chemicals and solvent were of analytical grade.

Cell proliferation reagent WST-1 (Roche Diagnostic, cat. N° 1644807, F. Hoffmann-La Roche Ltd, Diagnostics Division, Basel, Switzerland) was used as received. The rabbit corneal epithelial cell line (RCE) was obtained from the European Cell Culture Collection (N° 95081046, ECACC, Salisbury, UK) and was used for cytotoxicity test. Cell growth medium: Dulbecco's modified Eagle's medium (DMEM) with Ham's nutrient mixture F12 (1:1) added with L-glutamine (1% v/v), penicillin (100 IU/mL), streptomycin (0.1 mg/mL), amphotericin B (0.25 μg/mL), fetal bovine serum (15% v/v) (Gibco Invitrogen S.r.l., Milan, Italy), epidermal growth factor (10 ng/mL), and insulin (5 mg/mL) (Sigma Chemical Co., St Louis, MO).

2.2. Preparation of Quaternary Ammonium Chitosan-Methyl-β-Cyclodextrin conjugate (QA-Ch-MCD)

The procedure for the preparation of the alkylammonium chitosan-methylated-β-cyclodextrin conjugate is summarized in **Scheme 1**; the detailed overall representation of the reaction scheme is reported in the supplementary data file (**Scheme 1S**). Quaternary ammonium chitosan (QA-Ch60) was freshly prepared according to Zambito *et al* 2006 and 2008 [19, 20]. Briefly, CS (0.5g) was dissolved in 20 ml of 0.1 M hydrochloric acid (pH 4.7), overnight. DEAE-Cl (2.0 g) and 3 ml of 15% NaOH were added in sequence to the CS solution, under vigorous stirring at 60°C. The reaction proceeded for 2h at a controlled pH (pH=8). After cooling to room temperature, 1M HCl was used to adjust the solution to pH 7. The product was then dialyzed, filtered under vacuum, and lyophilized (VirTis AdVantage wizard 2.0, SP Scientific).

QA-Ch60 conjugation to MCD was performed in DMSO under inert N_2 atmosphere, through a three steps reaction. QA-Ch60 was dissolved at the concentration of 5% w/v. Two milliliters of this solution were added to 4ml of 0.28M HMDI. After adding 15μl TEA, the mix was kept at 70°C (N_2), for 3h under stirring. The obtained product

(QA-Ch-HMI) was precipitated in cold diethyl ether and washed twice, before being re-dispersed in 4ml of 0.14M MCD. The dispersion was placed at 70°C (N_2) and 40µl of TEA were added. After 3h, the reaction was quenched by dropping the mix into 10 ml of pre-warmed water (80°C) and left under stirring for 1h. The un-coupled MCD was removed by dialysis. The water-soluble product (QA-Ch-MCD) was recovered by lyophilizing the supernatant obtained after two centrifugation cycles (13000rpm x 30min, IEC MICROCL 17, Termo Fisher Scientific).
An intermediate product with pendant hexamethylene amine (QA-Ch-HMA) was prepared for characterization purposes. QA-Ch-HMA was obtained by re-dispersing the QA-Ch-HMI product, precipitated with Et_2O, in DMSO and directly quenching in 80°C water, followed by a similar work-up procedure as above.

2.3. Characterization

Attenuated total reflection, Fourier transformed infrared spectra (ATR/FT-IR) were recorded by using a Cary 660 series Agilent Technologies spectrophotometer, between 650 e 4000 cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) spectra of 1-2% samples in D_2O were recorded on INOVA 600 spectrometer Agilent Technologies at 25°C.

2.4. Phase-solubility studies

Phase-solubility studies of DEX in aqueous solutions with MCD, QA-Ch-MCD or mixtures of MCD and QA-Ch60 were performed by following a partially modified Jansook & Loftsson method [21]. Briefly, test solutions were sealed in glass vials with an excess amount of DEX and heated in autoclave (121 ◦C, 760 Torr) for 20 min. The dispersions were then equilibrated for 2h at 30°C under stirring at 144rpm, and afterwards at room temperature for 1h. The undissolved excess of DEX was removed by centrifugation at 13000rpm for 60min (IEC MICROCL 17, Termo Fisher Scientific). The aqueous samples tested included the following: plain MCD in the 0-2% range, MCD in the 0-2% range added with 0.1% of QA-Ch60, and the grafted polymer QA-Ch-MCD in the 0-1.2% range corresponding to 0-0.36% of conjugated MCD. Additionally, a stock solution containing 1.2% MCD and 2.8% QA-Ch60 (corresponding to a 30/70 MCD/QA-Ch60 wt ratio) was prepared and tested after diluting to 0.1-1.2%, on a MCD basis. The phase-solubility profiles were determined according to Higuchi & Connors 1965 and Brewster & Loftsson, 2007 [22, 23]. The stability constant (K) of the $MCD_1 \cdot DEX_m$ complex was determined from the linear phase-solubility diagrams (plots of the total drug solubility $[DEX]_t$ versus total MCD mM concentration $[MCD]_t$) according to the following equation (1):

$$K = (Slope)/(DEX_0^m\ (m\text{-}Slope)) \quad (1)$$

where Slope is the slope of the linear portion of the plot and DEX_0 stands for the intrinsic solubility of DEX in water.
For $MCD_1 \cdot DEX_1$ complexes, the complexation efficiency (CE) was defined as $[MCD_1 \cdot DEX_1]/[DEX_1]$ and calculated from equation (2) as:

$$CE = Slope/(1\text{-}Slope) \quad (2)$$

2.5. Analytical methods

The quantitative determination of DEX was performed spectroscopically at 242nm by using Shimadzu UV-2101 PC spectrophotometer (Shimadzu). An external calibration curve following linear regression was constructed in the range of concentrations 0.5-20μg/ml (R^2= 0.9976).

2.6. Differential scanning calorimetry (DSC)

Aqueous solutions of polymers, MCD and relevant DEX complexes were freeze-dried (VirTis Advantage wizard 2.0, SP Scientific). The lyophilized products (about 5mg samples) were subjected to DSC analysis in the 25-450°C temperature range at a heating rate of 10 °C min^{-1}, under a nitrogen flow rate of 20 ml min^{-1}, using a Pyris DSC 6, Perkin Elmer calorimeter. The acquired thermograms were processed by using Igor Pro 6.05 (WaveMetrics, Inc. Lake Oswego, OR, USA)

2.7. Rheological assessment of mucin-polymers adhesive bond strength

The method of Hassan and Gallo [24], consisting of the measurement of the viscosity changes induced in a mucin dispersion by the addition of the polymers, was essentially followed. Accordingly, the viscosity component due to mucoadhesion (η_b) was calculated from equation (3)

$$\eta_b = \eta_t - \eta_m - \eta_p \quad (3)$$

where η_t, η_m and η_p are the individual viscosity coefficients of the complete mixture (mucin plus polymer), mucin, and the polymer alone, respectively. Then, for the polymeric systems under study, the normalized parameter "adhesion index" (A.I.) = η_b / η_p was calculated. The viscosity measurements were carried out using a Rheostress RS 150 apparatus equipped with coaxial cylinders (Z40 and Z41), at shear rates ranging from 0 to 250 s^{-1}, at 32°C. The measurements were carried out on the following aqueous dispersions: i) 15% w/w mucin alone (HGM, η_m); ii) 0.1% w/w quaternized chitosan (QA-Ch60, η_p) or 0.1% w/w quaternized chitosan conjugated with MCD (QA-Ch-MCD, η_p); and iii) mixtures of HGM with either polymers at the same concentrations indicated above (η_t). For the polymeric dispersions that exhibited a pseudoplastic behavior, the apparent viscosity coefficient, η, was calculated for D = 1 s^{-1} from plots of τ vs D according to the equation (4)

$$\tau = a\, D^b \quad (4)$$

where *a* and *b* are experimentally determined values.

2.8. In vitro cytotoxicity evaluation

Cytotoxicity tests were carried out on RCE cells, using a WST-1 commercially available cell proliferation reagent. The assay is based on cleavage of the tetrazolium salt WST-1 by active mitochondria to produce a soluble coloured formazan salt. Since the conversion is operated only by viable cells, it directly correlates with the viable cell number. RCE cells, passage numbers 17-18, were plated at a density of 5 x 10^3 cells/well in a 96-well microtiter plate (Corning Costar®, Milan, Italy). Twenty four hours after plating, at 70% confluence and before the cultures became multilayered, the growth medium was removed and replaced with the test solutions (100 µl), consisting of QA-Ch-MCD solution prepared in growth medium. After 15 minutes exposure the test solutions were removed, the cells were washed twice with DMEM/F12, and 100 µl of fresh growth medium containing 1:10 of cell proliferation reagent WST-1 was added to each well. The cells were incubated for 2 h at 37 °C in a humidified atmosphere with 5% CO_2, then the microplate was thoroughly shaken for 1 min and the absorbance was measured at 450 nm using a microtiter reader (UVM340, Biochrom). The use of a two-hours incubation period was based on a series of preliminary experiments. The background absorbance was measured on wells only containing the

dye solution and the culture medium. The results were expressed as percent optical density of treated *vs*. control untreated wells [25].

3. Results

3.1 Preparation and characterization of QA-Ch-MCD

The QA-Ch-MCD derivative was prepared through a three-step reaction (**Scheme 1**, **Supplementary data file**), starting from the quaternized product QA-Ch60, i.e. *N,O*-[*N,N*-diethylaminomethyl(diethyldimethylene ammonium)$_n$ methyl]chitosan. The first step of the reaction consisted in the activation of QA-Ch60 by reacting it with HMDI, followed by precipitation in cold diethyl ether to remove the ungrafted spacer. The second step was MCD conjugation to the activated polymer via addition to the pendant isocyanate moiety. The third step consisted in quenching the reaction with water, which ended up with the liberation of carbon dioxide and the free terminal amine. The reaction was followed by ATR/FT-IR analysis and the quantification of the resulting functionalization was performed by ^{1}H NMR.

Figure 1 displays the ATR/FT-IR spectra of CS, QA-Ch60, QA-Ch-HMI and QA-Ch-MCD. With respect to CS, the ATR/FT-IR spectrum of QA-Ch60 showed a drastic reduction of the CS primary amine bending band ($\delta NH_2$1587 cm^{-1}) and a shift due to the overlapping with the amide II band of *N*-acetylglucosamine (1558 cm^{-1}). The spectrum also displayed an increase in multiplicity and intensity of the bands in the 3000-2800 cm^{-1} and 1470-1370cm^{-1} regions, due to the stretching and bending of the aliphatic pendant chain, respectively, and the presence of the tertiary amine stretching band at 1103 cm^{-1}. The occurred activation of QA-Ch60 with HMDI was confirmed by the presence of the bands at 2268, 1620, and 1580 cm^{-1}in the QA-Ch-HMI spectrum. The first one is specific for isocyanates and ascribable to the asymmetrical stretching vibrations of the –NCO, indicating the presence of isocyanate moieties available for the subsequent coupling with MCD. The other ones represent the ureic bridging moieties (ν C=O, δ N-H) between CS skeleton and spacer. The spectrum of the coupled product QA-Ch-MCD displayed both the ureic and urethane diagnostic bands of the linking moieties. In detail, the 3625-3100 cm^{-1} (ν OH and NH) region reflected the increase in hydroxyl moieties and free amine groups deriving from MCD and pendant uncoupled spacer, respectively. The alkyl ν C-H were found in the 3000-2850 cm^{-1} range whereas the carbamate and ureic characteristic bands were at 1704 and 1618cm^{-1}(ν C=O), and at 1537 and 1580 cm^{-1} (δ N-H), respectively. The latter and the δNH_2 of the uncoupled spacer were overlapping. Being an oligosaccharide, the presence of MCD in the QA-Ch-MCD spectrum was barely detectable. It can be associated with a shoulder (1082 cm^{-1}) of the main band at 1030cm^{-1}which seemed characteristic of the cyclodextrin and not of the chitosan derivative, as it fell in the range generally attributed to the ν_{CO} of the ring, namely, COH, COC and CH_2OH.

The quantification of the substitutions having occurred on QA-Ch-MCD was performed by ^{1}H NMR analysis and are shown in **Figure 2**. The spectrum of the conjugate was compared with the respective ones of the precursors, MCD and QA-Ch60, and of the product with pendant hexamethylene amine, QA-Ch-HMA (**Figures 1S**, **2S** and **3S**, **Supplementary data file**). Concerning the MCD, the methoxyl substitution (55%) was first verified by using the glucopyranose proton unit of the diagnostic signals at 4.93 ppm and 5.11 ppm, produced by the anomeric protons. The quantitative NMR analysis of QA-Ch60[19] led to the calculation of the degree of substitution (61.8%), the length of the alkylammonium chain (n=2), and also the content in methyl groups of ethyl moieties

bound to neutral and quaternized nitrogens in the spectral region 1.7-0.6 ppm. On this basis, the integrated area of the proton signal at 2.5 ppm (methylene bound to the terminal neutral nitrogen of the alkylammonium chain) in the ^{1}HNMR spectrum of QA-Ch-HMA and QA-Ch-MCD allowed us to evaluate the contribution of the central four methylene groups of the grafted spacer (about 50%), falling in the spectral region 1.7-0.6 ppm. The amount of coupled MCD was estimated by considering the terminal methylene protons at 2.5 ppm with respect to the diagnostic anomeric signals of the cyclodextrin at 5.13 and 4.95 ppm. The calculated substitution degrees are summarized in **Table 1.**

The applied coupling conditions resulted in a high degree of substitution of the HMDI spacer, consuming most of the residual free NH_2 of QA-Ch60. Accordingly, H-2 protons of QA-Ch-MCD were high-frequency shifted (about 3.4 ppm) with respect to the precursor (about 2.4 ppm). Additionally, the ATR/FT-IR analysis evidenced the predominance of ureic over carbamate bridging between QA-Ch60 and spacer. The quenching of the reaction limited the MCD coupling to 1/5 of the grafted spacer, with the conversion of the remaining –NCO moieties into pendant free amines. Although HMDI side reactions caused by possible water traces in the precursor polyols cannot be excluded, yet no biuret, allophanate or isocyanurates were detected.

The 10.6% degree of substitution of MCD on QA-Ch-MCD corresponds to 70/30 weight ratio of the precursors, i.e. QA-Ch-60/MCD. This ratio was used for the preparation of QA-Ch-60/MCD physical blends, which were submitted to the DEX solubility studies and the results compared to those obtained with the conjugated product (QA-Ch-MCD).

3.2 Solubility determination

Solubility profiles of DEX in aqueous MCD solutions and QA-Ch60/MCD blends are reported in **Figure 3a**.

DEX solubility increased linearly when plain MCD solutions were used. Differently, in presence of QA-Ch60/MCD blends the apparent solubility of DEX followed a linear profile for low MCD concentrations and drifted from linearity at higher MCD concentrations. This trend was first observed for solutions with constant QA-Ch60 concentrations (0.1%) and increasing MCD concentrations (0-2%). Then, a similar effect was detected when using solutions with increasing QA-Ch60 and MCD concentrations yet maintaining a constant 70/30 weight ratio. However, DEX solubility kept increasing up to 5mM MCD concentrations, before any deviation from linearity could be observed.

The use of QA-Ch-MCD for the formation of complexes with DEX determined a steep increase of DEX apparent solubility at low concentration of covalently bound MCD (**Figure 3b**). DEX solubility reached a maximum with QA-Ch-MCD concentrations corresponding to 2mM of MCD, and did not significantly increase further. This DEX apparent solubility value was twice the values achieved with MCD and MCD blends having the same MCD concentration (2mM).

The linear part of each phase-solubility profile was mathematically treated for the calculation of the stability constant (K) and the complexation efficiency (CE), where possible (**Table 2**). MCD formed 1:1 complexes with DEX, being the relevant slope less than unity. The calculated K and CE values increased with the addition of QA-Ch60, thus confirming the positive effect observed at low MCD concentrations. The slope of the linear part of the QA-Ch-MCD profile was more than unity and less than two, namely 1.1296 (R^2, 0.993). Hence, if a $MCD_1 \cdot DEX_2$ stoichiometry is supposed, the calculation of K from Equation (1) would lead to such an extremely high value as $35.9 \times 10^6 M^{-2}$ [22, 26]. The increase of DEX apparent solubility observed with the conjugated product, QA-Ch-

MCD, was confirmed by calculating the MCD/DEX molar ratio. The evaluation was performed by using CE for $K_{1:1}$ complexes and the concentrations at the plateau of the B_S profile [22]. The MCD/DEX molar ratio is more reliable, for comparative purposes, than the K values obtained from the phase solubility profiles, because the former is less sensitive to the experimental procedure than the latter. The enhancing effect of QA-Ch-60 on DEX complexation was proven by the reduction of the molar ratio from 2.19 to 1.88, corresponding to an overall MCD:DEX proportion of 2:1. Additionally, the use of QA-Ch-MCD polymer, with the covalently bound MCD, reduced the MCD/DEX value to 0.81, indicating an apparent 4:5 MCD/DEX ratio.

3.3 Thermal analysis of lyophilized complexes

The DSC thermograms of polymers and cyclodextrin are displayed in **Figure 4 (a)**. The quaternization of CS caused an anticipation of the degradation peak (exothermal onset at 286.8°C for CS *vs*. 218.3°C for QA-Ch60) which could be associated to the lower degree of crystallinity caused by the pendant alkyammonium chains [27]. Beside the endothermic peak of water evaporation, the MCD thermogram displays an endo/exo-thermic profile in the temperature range of 305-360 °C, previously associated to the melting (endo) and degradation (eso) transitions of cyclodextrins [28]. The physical blend QA-Ch60/MCD 70/30 determined a 9°C shift of the QA-Ch60 exothermal peak (onset at 227.5°C) associated to the degradation of the polymer. Unlike the physical blend, the QA-Ch-MCD thermogram was characterized by two exothermic peaks in the range 230-300°C (onset at 229.5°C and 262°C, respectively), followed by a wide exothermic peak with maximum at 362 °C.

The DSC thermograms of the lyophilized DEX complexes, with either the physical blend or QA-Ch-MCD are reported in **Figure 4 (b)**. The thermogram of the complex with the blend QA-Ch60/MCD 70/30 displayed an exothermal peak with onset at 226.4°C due to the degradation of the quaternized chitosan, followed by a small endothermic peak at 265°C, corresponding to the melting temperature of DEX. This small peak was not detected for the complexes of DEX with QA-Ch-MCD. The thermogram of these complexes displayed a broad exothermic peak with on set at 226.0°C.

3.4 Mucoadhesion and *In vitro* cytotoxicity evaluation

The results of the rheological studies aimed at evaluating the interactions between chitosan derivatives and HGM in aqueous dispersion are summarized in **Table 3**. Among the several methods employed to analyze the interaction of polymeric ingredients with a natural or artificial biological substrate, the rheological synergism method allows for the comparison between different viscous substances, by calculating the normalized parameter Adhesion Index (A.I.)[29]. This parameter represents a quali-quantitative measurement of the mucoadhesive properties of a substance. The test was used to compare the mucoadhesiveness of QA-Ch60, which has already been deeply investigated [8, 30, 31], with that of the MCD grafted product. The AI values calculated for both chitosan derivatives, i.e. QA-Ch60 and QA-Ch-MCD, are indicative of a meaningful interaction with mucin, typically ascribable to the quaternary ammonium moieties of the polymers. The conjugation to MCD determined a reduction of the A.I. value, as expected.

Concerning the *in vitro* cytotoxicity evaluation, the test was performed on RCE cell line, supposing a future deeper investigation for the application of the conjugate product as an excipient for eye drops. Cell viability was maintained above 80% for all QA-Ch-MCD tested concentrations (**Figure 5**). Furthermore, the EC_{50} value could

not be calculated because the cell viability data were remarkably higher than the 50% of controls, for all tested concentrations.

4. Discussion

CS-CD derivatives have commonly been obtained through glutaraldehyde crosslinking, CD coupling on carboxymethyl-chitosan through (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) *N*-hydroxysuccinimide (NHS) activation, or substitution of mono-6-deoxy-6-(p-toluenesulfonyl)-cyclodextrin, leading to the formation of three dimensional hydrogels, films, beads or particles [32-34]. In the case of water soluble CS-CD derivatives, the approaches described in the literature report on the quaternization of preformed CS-CD products by glycidyltrimethylammonium chloride [35]. Starting from our previous experience on QA-Ch60 and its derivatives, the water soluble CS-CD here described was obtained via direct conjugation of MCD to quaternized CS. HMDI has generally been applied as a crosslinking agent for the preparation of chitosan hydrogels[36], nevertheless, its use as spacer for water soluble CS derivatives has never been reported before. HMDI is a highly reactive coupling agent, generally applied for gel formation through chain covalent cross-linking. The formation of QA-Ch60 inter/intra chain cross-linking was hampered by using an excess of HMDI with respect to QA-Ch60 repeating units (RU) in the activation step [37]. Additionally, the formation of cross-linking via cyclodextrin bridging was limited by performing the second step on a washed QA-Ch-HMI intermediate and in presence of MCD molar excess. The molar ratio of the main reagents, i.e., RU/HMDI/MCD was set at 1/ 3.2 /1.6; corresponding to 1:5 reactive species in the first step (primary amines and hydroxyl groups of RU / isocyanate moieties) and 1:22 of pendant isocyanate moieties *vs* MCD primary hydroxyl groups, in the second step. Concerning the quenching step, the molar excess of water in presence of standard hydrolysis conditions (basic catalysis), determined the fast liberation of the free amine. The formation of additional ureic cross-linking between free amine and isocyanate could not be excluded, though. Future investigations shall concern the use of alternative methods for the isolation of the free amine [38]. Despite the applied reaction conditions, two types of products were isolated: a water-soluble QA-Ch-MCD conjugate and a not-soluble precipitate obtained as cross-linked by-product. This latter was eliminated by centrifugation. In the setting up of the reaction conditions, the durations of both the activation and grafting steps were optimized in order to achieve the maximum ratio between water-soluble/insoluble products. The applied conditions led to a wt% of 76.8 ± 2.7 of the soluble derivative, with respect to total reaction yield. The reaction was performed under basic catalysis, avoiding the use of stannous octoate, frequently adopted for NCO addition.
The water-soluble QA-Ch-MCD product was tested for the formation of macromolecular complexes with DEX and the obtained phase-solubility profile was compared to that recorded when physical blends of MCD and QA-Ch60 were used. According to the Higuchi-Connors classification system and in agreement with what already reported for other βCD derivatives [39], the DEX solubility profile in pure aqueous MCD solutions was classified as A_L type. The addition of QA-Ch60 to MCD solutions enhanced DEX apparent solubility at low MCD concentrations, but had an opposite effect at higher concentrations. Hence, the phase-solubility profile recorded changed from A_L to A_N type, when QA-Ch60/MCD blends were used. It is known that the apparent drug solubility is the sum of several contributions including the drug intrinsic solubility (DEX_0 was 0.19± 0.03 mM), soluble CD complexes, but also non-inclusion complexes deriving from CD self-association. In the case of QA-Ch60/MCD

blends, the presence of the polymer contributed by both non-specific adsorption of DEX and stabilization of MCD complexes until the occurrence of self-aggregation [26]. The use of the conjugated product QA-Ch-MCD determined a further change of the solubility profile into a B_S type. This B_S type profile consisted of a steep linear part with a slope greater than unity, followed by a plateau. The most important aspect was that the apparent MCD/DEX ratio changed from 2:1 to 4:5 when QA-Ch-MCD was used, indicating a strong interaction tendency. The occurrence of the plateau is mainly due to the loss of solubility of the QA-Ch-MCD/DEX complexes. Actually, according to the recorded data and in agreement with literature reports [40, 41], the cavity of βCD and its derivatives can host one DEX molecule. It appeared reasonable that for the QA-Ch-MCD polymer, the interaction with a second DEX molecule occurred outside the CD cavity, and that the added contribution from association resulted in a slope that is greater than unity. Moreover, this additional interaction could be responsible for the possible formation of hydrophobic clusters that may contribute to the reduced water solubility observed for QA-Ch-MCD/DEX complexes and typically correlated to the plateau of the Bs type profiles. Beside the use of quaternary ammonium chitosan, the choice of a 10-atom long spacer arm may have contributed to the increased DEX apparent solubility obtained with the conjugated product. Indeed, Buranaboripan *et al*. 2014[42] described two chitosan-βCD derivatives mainly differing in spacer length; in particular, a C0 and C4 (4-butylamido linking arm) spacers. Both derivatives were tested with 6-(p-toluidino)-2-napthalene-6-sulfonate (TNS) fluorescence probe and the studies were performed in acetate buffer to favor the aqueous solubility of the macromolecules. The C0 and C4 derivatives displayed a reduced K, compared to native βCD ($K=4000M^{-1}$), even though the longer spacer allowed for the maintenance of the complex stoichiometry ($K=2300\ M^{-1}$). These results suggested that the length of linking arm plays an important role in the formation of the inclusion complex. Additionally, Mateen and Hoare (2014)[43] described the reduction of K and CE of βCD complexes with DEX, with increasing βCD functionalization (adipic acid dihydrazide carboxymethyl functionalized βCD). However, the grafting of the fuctionalized βCD on polysaccharide (aldehyde-functionalized dextran), via a 12-atom long spacer, did not further interfere with complex formation. The authors concluded that immobilizing the CD derivative did not change its complexation behavior since no steric hindrance was closed to the hydrophobic binding pocket. Differently, in the present paper the covalent linkage of the MCD to QA-Ch60, through a 10-atom long spacer, affected the complex formation with DEX by increasing the apparent K, compared to native MCD at least for MCD concentrations up to 2mM. Despite the B_S profile, DEX solubility was ten-fold increased (1.97 ± 0.09 mM, at the plateau).

The results collected from the thermal analysis performed on both materials and lyophilized complexes are in agreement with what observed from the phase-solubility studies. The interaction between QA-Ch60 and MCD is supported by the 9°C shift of the on-set of QA-Ch60 degradation peak as well as by the absence of the melting/degradation profile of the cyclodextrin and the different profile of water evaporation peak. These observations imply a uniform molecular distribution of the MCD in the physical blend, causing a diverse water interaction, and polysaccharide supramolecular rearrangement. Concerning the conjugated QA-Ch-MCD product, it showed a higher thermal stability than the precursor QA-Ch60. Differently from what observed for the physical QA-Ch60/MCD blend, the wide exothermic profile suggests the presence of cyclodestrin rich domains, which could be correlated to the loss of solubility for the formation of clusters as observed for the B_S type profile. Additionally, the increased stability of the QA-Ch-MCD/DEX complexes is reflected in the absence of the DEX melting peak in the QA-Ch-MCD/DEX thermogram. Indeed, the region corresponding to the degradation profile of the polymer overlapped with the melting peak of DEX and could mask its detection. However, the lack of the

latter peak is also in agreement with the concomitant presence of inclusion complex interactions as well as with a molecular distribution of DEX, possibly associated to QA-Ch-MCD.

Concerning the mucoadhesive character, the QA-Ch-MCD product retains a meaningful interaction with mucin. Since mucoadhesion is generally mediated by electrostatic interactions between negatively charged mucin and cationic derivatives, the grafting of neutral cyclic oligosaccharides such as cyclodextrins is not contributing but hampering the strong muchoadhesion effect of the pristine quaternized QA-Ch60 polymer [16]. Additionally, in this specific case, the quaternary ammonium polymer (QA-Ch60) and its MCD conjugated derivative (QA-Ch-MCD) were tested at the same weight concentrations, which corresponded to different DEAE contents.

Concerning the *in vitro* cytotoxicity evaluation, cell viability was maintained above 80% for all QA-Ch-MCD tested concentrations, demonstrating that the polymer is safe and could be used as solubilizing excipient for ophthalmic use, at least up to a 10^{-2}% w/v concentration.

5. Conclusions

The developed QA-Ch-MCD appear promising for further applications as ophthalmic excipient since it showed a high ability to form a stable complex with the lipophilic drug dexamethasone, mucoadhesive features and cytocompatibility toward the RCE cell line. Direct MCD conjugation to QA-Ch60 led to a water-soluble product, which is generally not obtained by plain CS-CD derivatives. Furthermore, QA-Ch60 itself showed an enhancing effect toward MCD/DEX complexation, which was not obstructed when MDC was covalently linked through a long spacer arm. The strong interaction between QA-Ch60-MCD and DEX was also confirmed by DSC characterization, whereas the comparison between the stoichiometry of DEX / MCD and DEX / QA-Ch-MCD complexes has suggested for an interaction with a second DEX molecule outside the CD cavity. In this case, the possible formation of hydrophobic clusters could be the cause of the observed Bs type profile. The maximum DEX apparent solubility achieved is compatible with therapeutic applications of DEX, i.e. ophthalmic drops 0.05-0.2% presently commercialized as suspensions or solutions containing polysorbate surfactants. Thanks to the high interaction recorded between QA-Ch-MCD and DEX, the prepared derivative appears suitable for further processing into drug loaded CD-based supramolecular assemblies (e.g. nanocarriers or gels), as well as into 3D solid forms such as films or drug eluting medical devices [36, 32, 44, 34].

Acknowledgements

We gratefully acknowledge the financial support from P.R.A. 2017 by the University of Pisa.

References

1. Liu L, Zhu S. A study on the supramolecular structure of inclusion complex of β-cyclodextrin with prazosin hydrochloride. Carbohydrate Polymers. 2007;68(3):472-6. doi:10.1016/j.carbpol.2006.11.007.

2. Marques HMC, Hadgraft J, Kellaway IW. Studies of cyclodextrin inclusion complexes. I. The salbutamol-cyclodextrin complex as studied by phase solubility and DSC. International Journal of Pharmaceutics. 1990;63(3):259-66. doi:http://dx.doi.org/10.1016/0378-5173(90)90132-N.

3. Yang JS, Yang L. Preparation and application of cyclodextrin immobilized polysaccharides. J Mater Chem B. 2013;1(7):909-18. doi:10.1039/c2tb00107a.
4. Hansch C, Lao A, Hoekman D. Exploring QSAR. Hydrophobic, electronic and steric constants. . Crit Rev Toxicol 1995;25:67-89.
5. Rodríguez Villanueva J, Rodríguez Villanueva L, Guzmán Navarro M. Pharmaceutical technology can turn a traditional drug, dexamethasone into a first-line ocular medicine. A global perspective and future trends. International Journal of Pharmaceutics 2017;516:342-51.
6. Loftsson T, Stefánsson E. Cyclodextrins and topical drug delivery to the anterior and posterior segments of
the eye. Int J Pharmaceut 2017. doi: http://dx.doi.org/10.1016/j.ijpharm.2017.04.010.
7. Di Colo G, Zambito Y, Zaino C, Sansò M. Selected polysaccharides at comparison for their mucoadhesiveness and effect on precorneal residence of different drugs in the rabbit model. . Drug Development and Industrial Pharmacy. 2009;35:941-9.
8. Fabiano A, Chetoni P, Zambito Y. Mucoadhesive nano-sized supramolecular assemblies for improved pre-corneal drug residence time. Drug Development and Industrial Pharmacy. 2015;41:2069-76.
9. Piras AM, Sandreschi S, Maisetta G, Esin S, Batoni G, Chiellini F. Chitosan nanoparticles for the linear release of model cationic peptide. Pharmaceutical Research. 2015;32(7):2259-65. doi:10.1007/s11095-014-1615-9.
10. Dash M, Samal SK, Douglas TEL, Schaubroeck D, Leeuwenburgh SC, Van Der Voort P et al. Enzymatically biomineralized chitosan scaffolds for tissue-engineering applications. Journal of Tissue Engineering and Regenerative Medicine. 2017;11(5):1500-13. doi:10.1002/term.2048.
11. Piras AM, Maisetta G, Sandreschi S, Gazzarri M, Bartoli C, Grassi L et al. Chitosan nanoparticles loaded with the antimicrobial peptide temporin B exert a long-term antibacterial activity in vitro against clinical isolates of Staphylococcus epidermidis. Frontiers in Microbiology. 2015;6(APR). doi:10.3389/fmicb.2015.00372.
12. Henriksen I, Green KL, Smart JD, Smistad G, Karlsen J. Bioadhesion of hydrated chitosans: An in vitro and in vivo study. International Journal of Pharmaceutics. 1996;145(1–2):231-40. doi:http://dx.doi.org/10.1016/S0378-5173(96)04776-X.
13. Lehr C-M, Bouwstra JA, Schacht EH, Junginger HE. In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers. International Journal of Pharmaceutics. 1992;78(1–3):43-8. doi:http://dx.doi.org/10.1016/0378-5173(92)90353-4.
14. Tamer TM, Hassan MA, Omer AM, Valachova K, Eldin MSM, Collins MN et al. Antibacterial and antioxidative activity of O-amine functionalized chitosan. Carbohydr Polym. 2017;169:441-50. doi:10.1016/j.carbpol.2017.04.027.
15. Prabaharan M, Mano JF. Chitosan derivatives bearing cyclodextrin cavitiesas novel adsorbent matrices. Carbohydrate Polymers. 2006;63(2):153-66. doi:http://dx.doi.org/10.1016/j.carbpol.2005.08.051.
16. Chaleawlert-umpon S, Nuchuchua O, Saesoo S, Gonil P, Ruktanonchai UR, Sajomsang W et al. Effect of citrate spacer on mucoadhesive properties of a novel water-soluble cationic β-cyclodextrin-conjugated chitosan. Carbohydrate Polymers. 2011;84(1):186-94. doi:http://dx.doi.org/10.1016/j.carbpol.2010.11.017.
17. Laine V, Coste-Sarguet A, Gadelle A, Defaye J, Perly B, Djedaini-Pilard F. Inclusion and solubilization properties of 6-S-glycosyl-6-thio derivatives of [small beta]-cyclodextrin. Journal of the Chemical Society, Perkin Transactions 2. 1995(7):1479-87. doi:10.1039/P29950001479.
18. Zambito Y, Felice F, Fabiano A, Di Stefano R, Di Colo G. Mucoadhesive nanoparticles made of thiolated quaternary chitosan crosslinked with hyaluronan. Carbohydrate Polymers. 2013;92(1):33-9. doi:http://dx.doi.org/10.1016/j.carbpol.2012.09.029.

19. Zambito Y, Uccello-Barretta G, Zaino C, Balzano F, Di Colo G. Novel transmucosal absorption enhancers obtained by aminoalkylation of chitosan. European Journal of Pharmaceutical Sciences. 2006;29(5):460-9. doi:http://dx.doi.org/10.1016/j.ejps.2006.09.001.
20. Zambito Y, Zaino C, Uccello Barretta G, Balzano F, Di Colo G. Improved synthesis of quaternary ammonium–chitosan conjugates (N+–Ch) for enhanced intestinal drug permeation. Eur J Pharm Sci 2008;33:343-50.
21. Jansook P, Loftsson T. CDs as solubilizers: Effects of excipients and competing drugs. International Journal of Pharmaceutics. 2009;379:32-40.
22. Brewster ME, Loftsson T. Cyclodextrins as pharmaceutical solubilizers. Advanced Drug Delivery Reviews. 2007;59:645-66.
23. Higuchi T, Connors KA. Phase-solubility techniques. . Adv Anal Chem Instrum 1965;4:117-212.
24. Hassan E, Gallo J. A simple rheological method for the in vitro assessment of mucin-polymer bioadhesive bond strength. Pharm Res 1990;7:491-5.
25. Chetoni P, Monti D, Tampucci S, Matteoli B, Ceccherini-Nelli L, Subissi A et al. Liposomes as a potential ocular delivery system of distamycin A. International Journal Pharmaceutics. 2015;492:120-6.
26. Loftsson T, Brewster ME. Pharmaceutical applications of cyclodextrins: basic science and product development. Journal of Pharmacy and Pharmacology. 2010;62:1607-21.
27. Gonil P, Sajomsang W, Ruktanonchai UR, Pimpha N, Sramala I, Nuchuchua O et al. Novel quaternized chitosan containing β-cyclodextrin moiety: Synthesis, characterization and antimicrobial activity. Carbohydrate Polymers. 2011;83(2):905-13. doi:10.1016/j.carbpol.2010.08.080.
28. Song LX, Teng CF, Xu P, Wang HM, Zhang ZQ, Liu QQ. Thermal decomposition behaviors of β-cyclodextrin, its inclusion complexes of alkyl amines, and complexed β-cyclodextrin at different heating rates. Journal of Inclusion Phenomena and Macrocyclic Chemistry. 2007;60(3-4):223-33. doi:10.1007/s10847-007-9369-1.
29. Burgalassi S, Nicosia N, Monti D, Falcone G, Boldrini E, Chetoni P. Larch arabinogalactan for dry eye protection and treatment of corneal lesions: investigations in rabbits. J Ocul Pharmacol Ther. 2007;23(6):541-50.
30. Rassu G, Gavini E, Jonassen H, Zambito Y, Fogli S, Breschi MC et al. New chitosan derivatives for the preparation of rokitamycin loaded microspheres designed for ocular or nasal administration. Journal of Pharmaceutical Sciences. 2009;98(12):4852-65. doi:10.1002/jps.21751.
31. Uccello-Barretta G, Balzano F, Aiello F, Senatore A, Fabiano A, Zambito Y. Mucoadhesivity and release properties of quaternary ammonium-chitosan conjugates and their nanoparticulate supramolecular aggregates: An NMR investigation. International Journal of Pharmaceutics. 2014;461(1-2):489-94. doi:10.1016/j.ijpharm.2013.12.018.
32. Kono H, Teshirogi T. Cyclodextrin-grafted chitosan hydrogels for controlled drug delivery. International Journal of Biological Macromolecules. 2015;72:299-308.
33. Wilson LD, Pratt DY, Kozinski JA. Preparation and sorption studies of β-cyclodextrin–chitosan–glutaraldehyde terpolymers. Journal of Colloid and Interface Science. 2013;393:271-7. doi:http://dx.doi.org/10.1016/j.jcis.2012.10.046.
34. Yuan Z, Yeb Y, Gao F, Yuan H, Lan M, Lou K et al. Chitosan-graft-b-cyclodextrin nanoparticles as a carrier for controlled drug release. International Journal of Pharmaceutics. 2013;446:191-8.
35. Sajomsang W, Nuchuchua O, Saesoo S, Gonil P, Chaleawlert-umpon S, Pimpha N et al. A comparison of spacer on water-soluble cyclodextrin grafted chitosan inclusion complex as carrier of eugenol to mucosae. Carbohydrate Polymers. 2013;92(1):321-7. doi:http://dx.doi.org/10.1016/j.carbpol.2012.08.106.

36. Concheiro A, Alvarez-Lorenzo C. Chemically cross-linked and grafted cyclodextrin hydrogels: From nanostructures to drug-eluting medical devices. Advanced Drug Delivery Reviews. 2013;65:1188-203.
37. Salmaso S, Semenzato A, Bersani S, Matricardi P, Rossi F, Caliceti P. Cyclodextrin/PEG based hydrogels for multi-drug delivery. Int J Pharm. 2007;345(1-2):42-50. doi:10.1016/j.ijpharm.2007.05.035.
38. Ma B, Lee W-C. A modified Curtius reaction: an efficient and simple method for direct isolation of free amine. Tetrahedron Letters. 2010;51:385-6.
39. Liu X-M, Lee H-T, Reinhardt RA, Marky LA, Wang D. Novel biomineral-binding cyclodextrins for controlled drug delivery in the oral cavity. Journal of Controlled Release. 2007;122:54-62.
40. Magnusdottir A, Másson M, Loftsson T. Self Association and Cyclodextrin Solubilization of NSAIDs. Journal of Inclusion Phenomena (2002) 44: 213 doi:101023/A:1023079322024. 2002;44:213-8. doi:10.1023/A:1023079322024.
41. Vianna RFL, Bentley MVLB, Ribeiro G, Carvalho FS, Neto AF, de Oliveira DCR et al. Formation of cyclodextrin inclusion complexes with corticosteroids. International Journal of Pharmaceutics. 1998;167:205-13.
42. Buranaboripan W, Lang W, Motomura E, Sakairi N. Preparation and characterization of polymeric host molecules,b-cyclodextrin linked chitosan derivatives having different linkers. International Journal of Biological Macromolecules. 2014;69:27–34.
43. Mateen R, Hoare T. Carboxymethyl and hydrazide functionalized beta-cyclodextrin derivatives: a systematic investigation of complexation behaviours with the model hydrophobic drug dexamethasone. Int J Pharm. 2014;472(1-2):315-26. doi:10.1016/j.ijpharm.2014.06.046.
44. Simões SMN, Rey-Rico A, Concheiro A, Alvarez-Lorenzo C. Supramolecular cyclodextrin-based drug nanocarriers. Chemical Communications. 2015;51(29):6275-89. doi:10.1039/c4cc10388b.

Scheme 1 : Reaction scheme for the preparation of the alkylammonium chitosan-methylated-β-cyclodextrin conjugate. The scheme includes the quaternization of CS to QA-Ch60, followed by the activation with HMDI (QA-Ch-HMI), the grafting of MCD (conjugation step) and the quenching of the unreacted isocyanate terminal to QA-Ch-MCD. A detailed reaction scheme, including also the polymer with pendant hexamethylamine (QA-Ch-HMA), is reported in the supplementary data file (Scheme 1S).

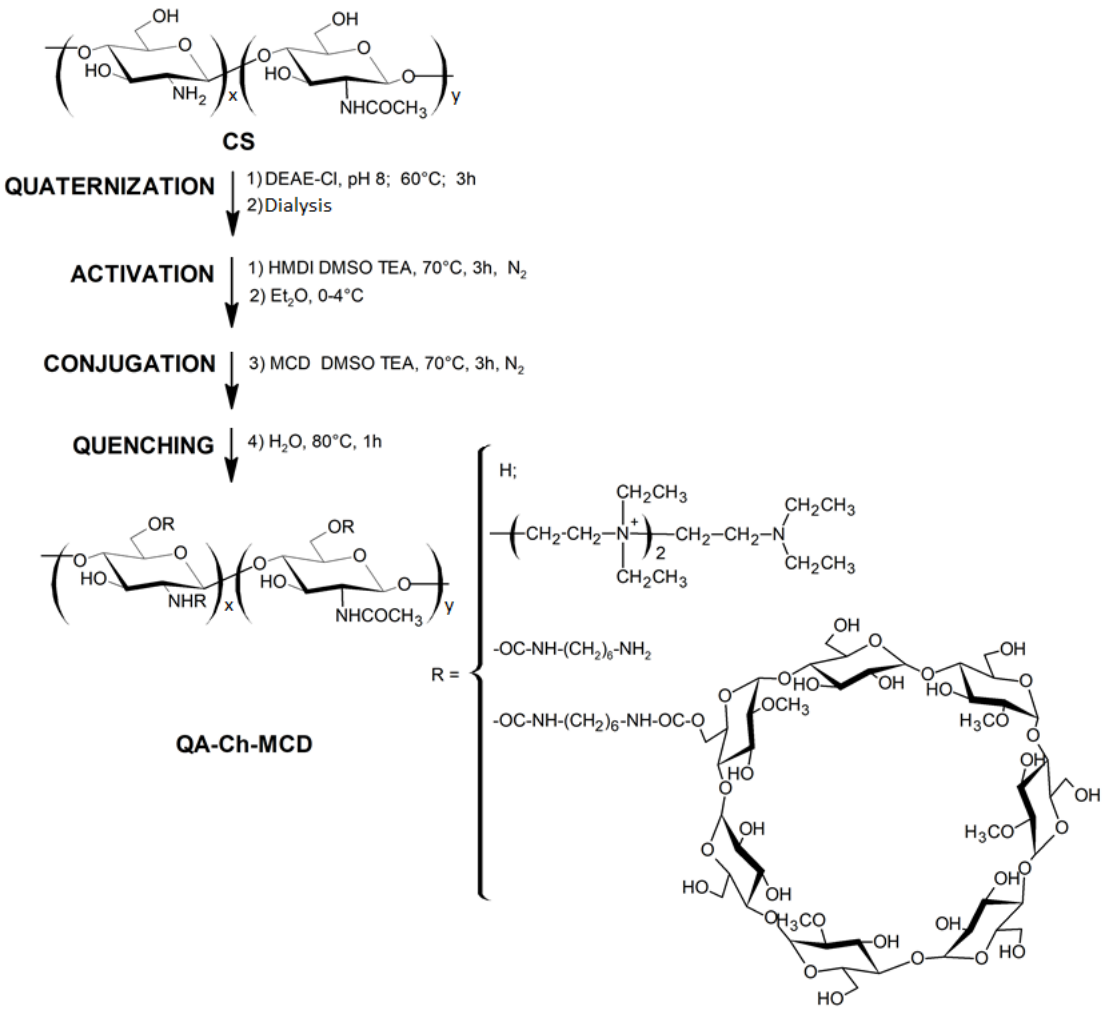

Fig. 1 ATR/FT-IR (A%) spectra of CS, QA-Ch60, QA-Ch-HMI and QA-Ch-MCD.

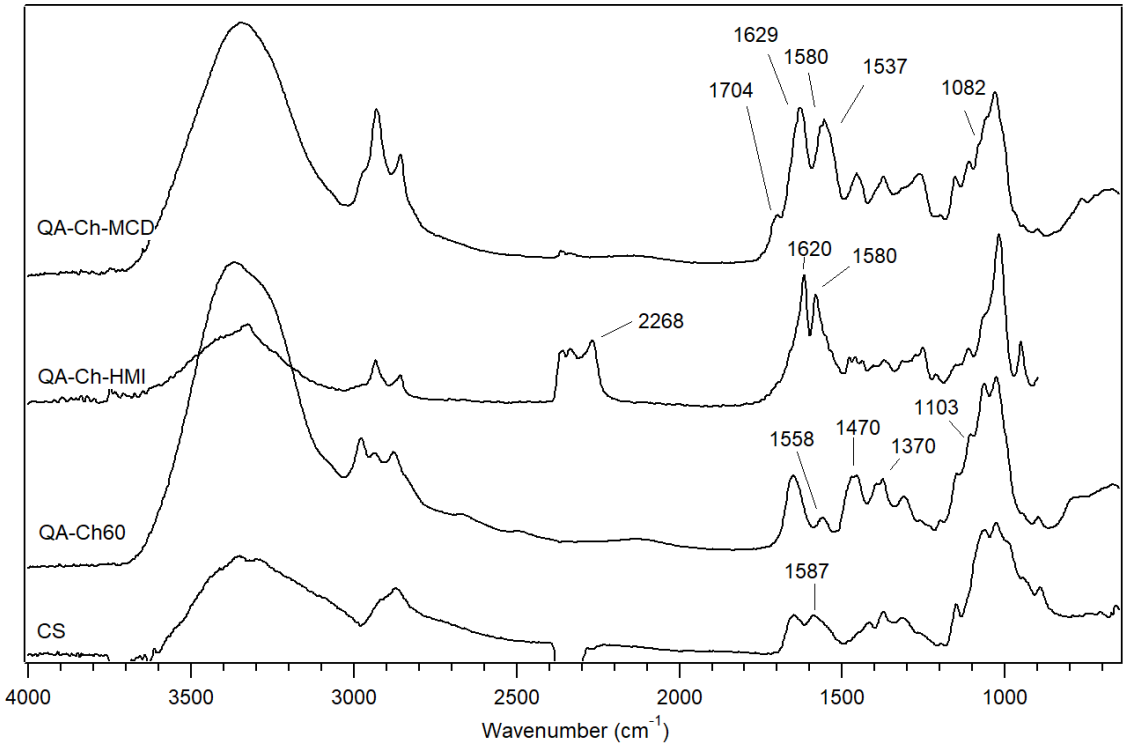

Fig. 2 ^{1}H NMR (600 MHz, D_2O) spectrum of QA-Ch-MCD. δ = 5.13 and 4.95 (s, MDC anomerics), 4.2-2.6 (m, protons of the pyranosidic ring, methylene protons of pendant quaternized chains –C**H$_2$**C**H$_2$**N$^+$(C**H$_2$**CH$_3$)$_2$C**H$_2$**- and –C**H$_2$**N(CH$_2$CH$_3$)$_2$, methylene protons closed to the N of the spacer -NH**CH$_2$**(CH$_2$)$_4$**CH$_2$**NH$_2$); 2.5 (s, methylene protons of not protonated N of the DEAE pendant –CH$_2$N(**CH$_2$**CH$_3$)$_2$), 2.0 (s, methyl protons of *N*-acetylglucosamine), 1.7-1.1 (m, methyl protons of the ethyl moieties closed to ammonium pendants N$^+$(CH$_2$C**H$_3$**)$_2$ and methylene protons of the central part of spacer chain -NHCH$_2$(**CH$_2$**)$_4$CH$_2$NH$_2$), 1.0-0.7 ppm (m, methyl protons of the terminal part of DEAE chains -N(CH$_2$C**H$_3$**)$_2$).

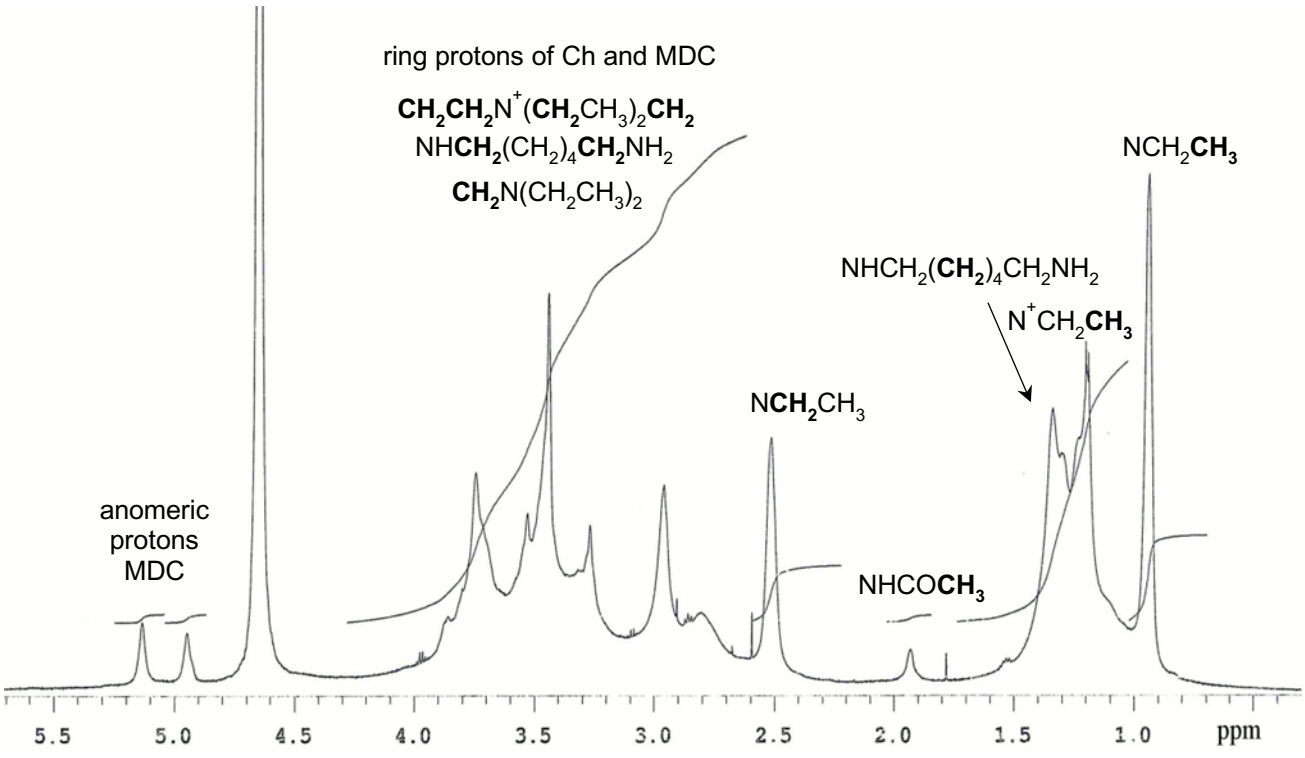

Fig. 3 Solubility profiles of DEX in presence of a) MCD, QA-Ch60/MCD 73/30 blend, and MCD added with 0.1% QA-Ch60 (QA-Ch60/MCD 0.1%/0-2%); b) QA-Ch-MCD (MCD covalently conjugated to QA-Ch-60).

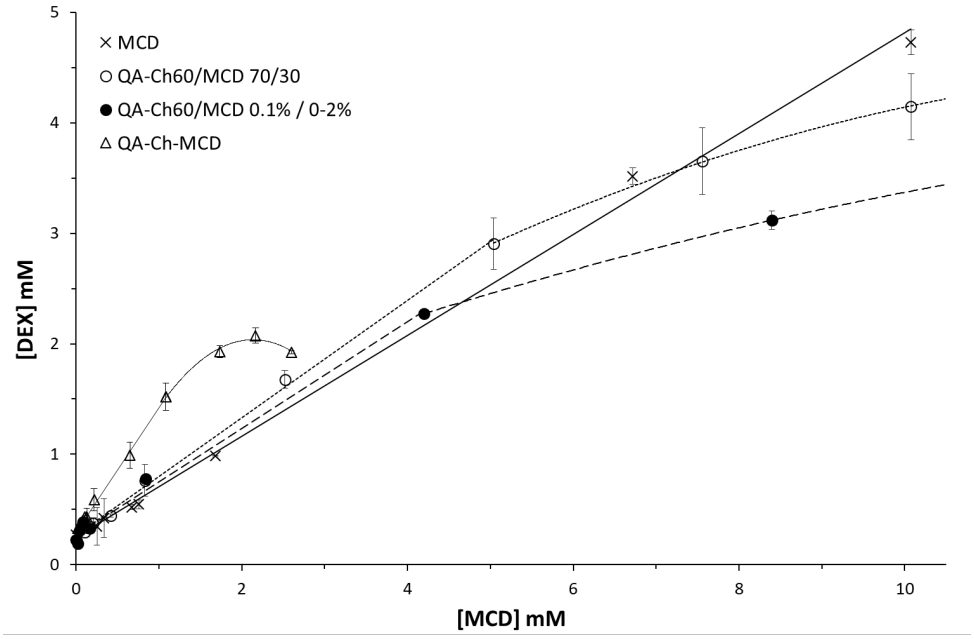

Fig. 4 DSC thermograms of: a) CS, QA-Ch60, MCD, QA-Ch-MCD, and QA-Ch60/MCD 70/30 blend; b) lyophilized DEX and its complexes with QA-Ch-MCD, and QA-Ch60/MCD 70/30 blend

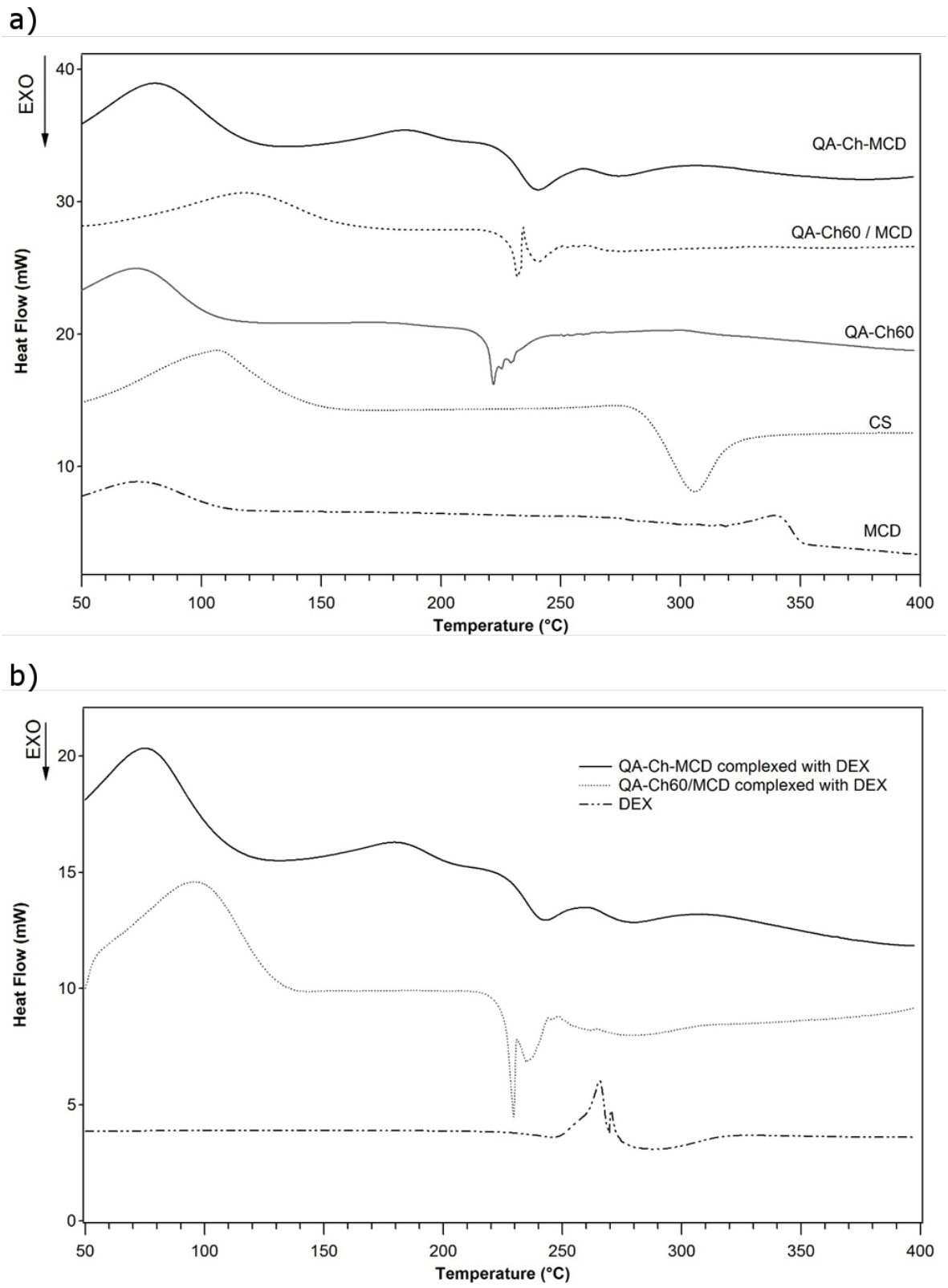

Fig. 5 *In vitro* citotoxycity evaluation of QA-Ch-MCD performed on RCE cell line.

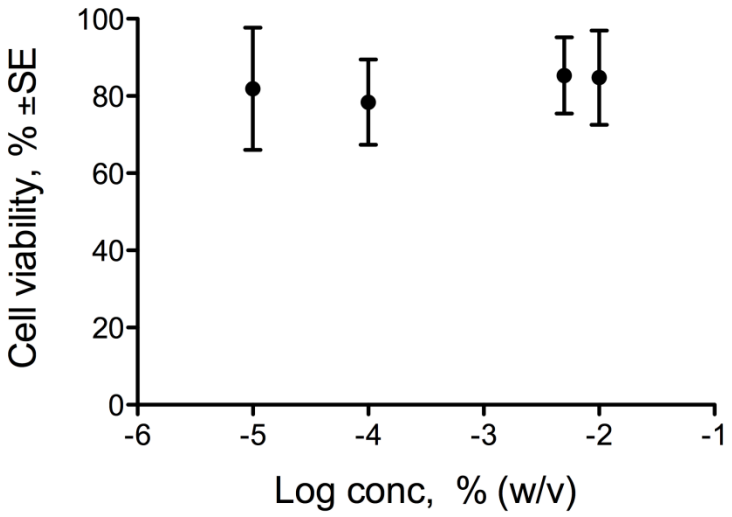

Table 1

Degree of substitution for precursors and products as determined by ^{1}H NMR. Percentage calculated with respect to sugar rings

	Substituent	% DS
MCD	$-CH_3$	55.5
QA-Ch60	$-COCH_3$	15.2
	$-CH_2[CH_2N^+(CH_2CH_3)_2\ CH_2]_n-CH_2N(CH_2CH_3)_2$	61.8; n = 2
QA-Ch-HMA	$-COCH_3$	15.2
	$-CH_2[CH_2N^+(CH_2CH_3)_2\ CH_2]_n-CH_2N(CH_2CH_3)_2$	61.9; n = 2
	$-CONHCH_2(CH_2)_4CH_2NH_2$	52.0
QA-Ch-MCD	$-COCH_3$	15.2
	$-CH_2[CH_2N^+(CH_2CH_3)_2\ CH_2]_n-CH_2N(CH_2CH_3)_2$	61.7; n = 2
	$-CONHCH_2(CH_2)_4CH_2NH-$	48.9
	–MeβCD	10.6

Table 2

Characteristics of DEX/MCD inclusion complexes obtained by using water solutions of MCD alone, MCD in blends with QA-Ch60, and MCD covalently conjugated to the quaternary ammonium chitosan (QA-Ch-MCD). Stability constant (K), complexation efficiency (CE) and molar ratio, slope of the linear portion of the phase-solubility studies. DEX intrinsic solubility was determined as $DEX_0 = 0.19mM$

	Complex $MCD_n \cdot DEX_m$			Linear regression	
	K (M^{-1})	**CE**	**Molar ratio MCD/DEX**	**Slope**	**R^2**
MCD	4428	0.84	2.19	0.4569	0.997
QA-Ch60/MCD, 0.1%/0–2%	4876	0.93	2.08	0.4809	0.991
QA-Ch60/MCD, 70/30	6289	1.13	1.88	0.5312	0.998
QA-Ch-MCD	35.9×10^{6}[a]	–	0.81[b]	1.1296	0.993

[a]According to the general equation $K = (Slope)/(DEX_0^m \cdot (m\text{-}Slope))$ and considering $1 < Slope < 2$, the hypothetical $MCD_1 \cdot DEX_m$ stoichiometry could be 1:2 and the resulting K calculated as $35.9 \times 10^6\ M^{-2}$

[b]Calculated from the supposed plateau of the B_S profile [20]

Table 3

Rheological assessment of mucin-polymers adhesive bond strength

	Viscosity (mPa•s)				A.I.
	η_m	**η_p**	**η_t**	**η_b**	**η_b/η_p**
HGM	44.49	–	–	–	–
QA-Ch60	–	3.71	–	–	–
QA-Ch-MCD	–	5.52	–	–	–
HGM/QA-Ch60	–	–	73.69	25.49	6.87
HGM/QA-Ch-MCD	–	–	57.97	7.96	1.44