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Research paper

Enhanced delivery of daidzein into fibroblasts and neuronal cells with cationic derivatives of gamma-cyclodextrin for the control of cellular glycosaminoglycans

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ABSTRACT

Two cationic derivatives of γ-cyclodextrin (GCD) were synthesized by functionalization with glycidyltrimethylammonium chloride (GTMAC) and ethylenediamine (EDA). Both these derivatives (GCD-GTMAC and GCD-EDA) have been shown to interact strongly with anionic biopolymers, unfractionated heparin (UFH) and mucin, the latter showing their mucoadhesive properties. They form inclusion complexes with daidzein (DAI), an isoflavone displaying a multitude of physiological effects, much more efficiently than the unmodified GCD. It was also shown that the complexes of these GCD derivatives with DAI and Nile Red penetrate human fibroblasts and murine hippocampal neuronal cells indicating that cationic GCD derivatives can be considered as potential delivery systems for isoflavones and other poorly water soluble compounds. Moreover, it was found that DAI delivered in cationic GCD complexes decreased the level of the cellular glycosaminoglycans (GAGs) in normal fibroblasts suggesting their possible application in the control of GAGs in mucopolysaccharidoses, lysosomal storage diseases caused by pathological accumulation of GAGs in the cells.

1. Introduction

Cyclodextrins are a family of oligosaccharides, whose molecules are made up of glucose units bound together to form a circle with a hydrophobic cavity in the center [22]. The most common structures are constructed with 6 (alpha), 7 (beta), or 8 (gamma) glucose units [22,36]. One of the most characteristic features of these compounds is the ability to form inclusion complexes [33]. The cavity can be occupied by hydrophobic molecules whose solubility thereby significantly increases. Therefore cyclodextrins are often used in food, pharmaceutical [26], drug delivery [40,13] , and chemical industries, as well as in agriculture and textile industry [2].

Chemical modification of cyclodextrin by substitution with ionized groups may further enhance the solubility of their complexes. It may also improve the efficiency of the formation of the inclusion complexes and their bioavailability [3,43]. In particular, cationic modification of the cyclodextrins may result in increased mucoadhesion [5], which is a desired property of transmucosal delivery systems, including oral ones. Cationic cyclodextrins were found to be efficient cell penetrating agents [45], therefore they may be used in drug delivery [38] and gene therapy [25], like other polycationic vectors [47,50].

The purpose of this research was to study the application of two derivatives of γ-cyclodextrin (GCD) as potential drug delivery systems. We have used them for the delivery of daidzein (DAI), one of natural isoflavones occurring abundantly in leguminous plants and showing many beneficial pharmaceutical properties [41]. DAI is a free radical scavenger and antioxidant [8] which shows anticancer [41] and neuroprotective activity [15]. However, the DAI property, which we were most interested in, was its ability to inhibit the cellular synthesis of glycosaminoglycans (GAGs). This is why daidzein and other isoflavones are considered as possible drugs for the treatment of mucopolysaccharidoses [21,1,31]. However, the main problem with the therapeutical use of DAI is its very poor solubility in water and hence a very low bioavailability.

In this paper we report studies which aimed at the development of a delivery system for DAI using cationically-modified cyclodextrins. So far there have been only a few reports on solubilization of DAI [29] or its derivatives [44] using cyclodextrins. Our studies were stimulated by the recent report showing that β-cyclodextrin may enhance the transport of DAI and genistein, another isoflavone, through the Caco 2 cell monolayer [6].

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The important aspects of this work are that, on one hand, we have demonstrated that the complexes of the obtained cyclodextrins with DAI and Nile Red (NR), a fluorescent dye forming inclusion complexes with cyclodextrins [42], penetrate into human dermal fibroblasts and into murine hippocampal neuronal cells in vitro. On the other hand, we have shown that thus delivered DAI significantly decreases GAGs level in healthy human dermal fibroblasts at concentrations at which it does not impair cell viability. These findings indicate that the prepared cationic cyclodextrins may be potential drug delivery systems in the therapy of MPS, in particular, in the MPS types severely affecting nervous systems, such as Sanfilippo disease.

2. Experimental section

2.1. Materials

Daidzein (DAI, >98%, Sigma–Aldrich), γ-cyclodextrin (GCD, ⩾90.0% cyclodextrin basis (HPLC), Wacker Chemie AG, Burghause, Sigma–Aldrich), heparin sodium salt from bovine intestinal mucosa (Sigma), mucin from porcine stomach (Type III, bound sialic acid 0.5–1.5%, partially purified powder, Sigma), glycidyltrimethylammonium chloride (GTMAC, technical ⩾90%, Sigma–Aldrich), ethylenediamine (EDA, analytical grade, POCh), Crystal Violet (CV, ACS reagent, ⩾90.0% anhydrous basis, Sigma–Aldrich), Alcian Blue 8GX (AB, for microscopy, Sigma–Aldrich), Nile Red (NR, for microscopy, Sigma–Aldrich), citric acid (analytical grade, POCh), sodium citrate (analytical grade, POCh), sodium dodecyl sulfate (SDS, BioReagent, ⩾98.5%, Sigma–Aldrich), potassium chloride (analytical grade, POCh), potassium dihydrogen phosphate (analytical grade, POCh), disodium hydrogen phosphate (analytical grade, POCh), sodium chloride (analytical grade, POCh), *p*-toluenesulfonyl chloride (TsCl, 99%, Fluka), polyornithine (Sigma–Aldrich), acetone (analytical grade, POCh), ethanol (96%, analytical grade, POCh), proteinase K (lyophilized powder, BioUltra, ⩾30 units/mg protein, Sigma–Aldrich) were used as received. The B27 and neurobasal medium were obtained from Gibco (Grand Island, NY, USA). Pyridine (analytical grade, POCh) was dried over 3 Å molecular sieves predesiccated in 120 °C for 24 h. Water was distilled twice and deionized using the Millipore SIMPLCITY system. Culture plates were from TPP Techno Plastic Products AG (Trasadingen, Switzerland).

2.2. Apparatus

UV–Vis absorption spectra were recorded using a HP8452A diode-array spectrophotometer in 1-cm optical path quartz cells. The size of the aggregates in the aqueous suspension was determined using Malvern Instruments Ltd. Nano ZetaSizer. FTIR spectra were obtained using a Bruker IFS 48 spectrometer. NMR spectra were measured in D_2O using a Bruker AMX 500 spectrometer. Elemental analysis was performed using a Vario Micro CHNS elemental analyzer (Elementar).

2.3. Synthesis of GCD substituted with GTMAC (GCD-GTMAC)

To the solution of 0.40 g (0.31 mmol) of GCD in 20 ml of distilled water 80 mg (2.0 mmol) of NaOH and 4.8 ml (36 mmol) of GTMAC were added. The mixture was heated up to 60 °C and the reaction was carried out for 2 h with stirring. The mixture was then neutralized to pH = 7 using HCl and 60 ml of acetone was added. The resulting suspension was centrifuged at 9000 rpm (8586*g*) for 1 min. The supernatant was removed and 60 ml of acetone was added. Washing with acetone was repeated three times and the resulting precipitate was dried in vacuum for 4 h to remove acetone. The dry solid was triturated in a mortar, suspended in 50 ml of ethanol, and shaken for 24 h. Ethanol was removed by decantation and the precipitate was dried in vacuum for 12 h. Yield: 60%.

2.4. Synthesis of GCD substituted with EDA (GCD-EDA)

In 100 ml of dry pyridine 13.0 g (10 mmol) of GCD and 17.5 g (92 mmol) of TsCl were dissolved using a Schlenk flask under nitrogen. The liquid was stirred at room temperature for 24 h. Then, pyridine was evaporated using a rotary evaporator and the remaining viscous fluid was poured into cold water. The resulting precipitate was collected by filtration under reduced pressure. The precipitate was washed three times with 50 ml aliquots of water at room temperature, dried under vacuum for 12 h, and dissolved in 50 ml of EDA. The resulting mixture was reacted at 40 °C for 24 h with stirring. Then, EDA was evaporated using rotary evaporator and the viscous fluid was poured into cold acetone. The precipitate was washed three times with 50 ml portions of acetone at room temperature and dried under vacuum for 12 h. Yield: 40%.

2.5. Preparation of GCD-GTMAC complex with DAI (GCD-GTMAC/DAI)

10 mg (0.039 mmol) of DAI was dissolved in 10 ml of methanol. Then, 100 mg of GCD-GTMAC (0.040 mmol) dissolved in 1 ml water was added to this solution and left on a shaker for 24 h at 25 °C. Next, methanol was removed on a rotary evaporator and the resulting product was freeze-dried. The complex was easily soluble in water forming a clear colorless solution in the concentration range applied.

2.6. Preparation of GCD-GTMAC complex with NR (GCD-GTMAC/NR)

2.7 mg (0.0084 mmol) of NR was dissolved in 15 ml of methanol. Then, 37 mg (0.015 mmol) of GCD-GTMAC dissolved in 0.5 ml of water was added to this solution and left on a shaker for 24 h at 25 °C. Next, methanol was removed on a rotary evaporation and the resulting product was dried in a vacuum oven at 60 °C. The complex was easily soluble in water forming a clear colorless solution in the concentration range applied.

2.7. Preparation of GCD-EDA complex with DAI (GCD-EDA/DAI)

To 10 ml of water 20 mg (0.079 mmol) of DAI and 80 mg (0.052 mmol) of GCD-EDA were added and the resulting suspension was shaken until transparent solution was obtained. Then, the liquid was centrifuged at 4000 rpm (1696*g*) for 2 min, the supernatant was separated, and the solvent was removed by freeze-drying. The complex was easily soluble in water forming a clear colorless solution in the concentration range applied.

2.8. Preparation of GCD-EDA complex with NR (GCD-EDA/NR)

To 2 ml of water 2 mg (0.0063 mmol) of NR and 8 mg (0.0052 mmol) of GCD-EDA were added and the suspension formed was left on a shaker for 24 h at 25 °C. Then, the obtained mixture was centrifuged at 4000 rpm (1696*g*) for 2 min, the supernatant was separated, and the solvent was removed by freeze-drying. The complex was easily soluble in water forming a clear colorless solution in the concentration range applied.

2.9. DAI solubilization by GCD and its cationic derivatives

In 2 ml of water 2 mg (0.0079 mmol) of DAI and increasing amounts of GCD or its cationic derivatives (0–16 mg, 0–12.3 mmol of GCD, 0–29 mg, 0–11.6 mmol of GCD-GTMAC, and 0–6 mg,

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0–3.6 mmol of GCD-EDA) were suspended. The suspension was shaken for 2 h at 56 °C and sonicated for 5 min every 30 min. After 5 sonication cycles the suspension was centrifuged at 4000 rpm (1696*g*) for 2 min and the absorption and fluorescence spectra of the supernatant were measured at λ_{ex} = 305 nm. The concentration of complexed DAI was calculated based on the calibration curve obtained using DAI solutions in methanol.

2.10. Determination of uncomplexed UFH

The concentration of free UFH, which was not complexed by GCD cationic derivatives, was determined by using an Azure A spectrophotometric method, as described earlier (see for example [20]. The mixture of UFH (c = 0.2 mg/ml) and GCD derivatives at varying concentrations was shaken for 10 min and the mixture was centrifuged at 4000 rpm (1696*g*) for 10 min to separate the colloidal UFH – cationic GDC complex. The concentration of UFH uncomplexed by cationic GCDs in the supernatant was determined by measuring the absorbance at 630 nm, characteristic of the monomeric Azure A molecules. In the presence of free UFH in solution Azure A molecules form dimers absorbing at 513 nm and the absorption band with a maximum at 630 nm decreases due to the falling concentration of monomeric Azure A molecules. Thus, the absorbance at 630 nm is proportional to the free UFH concentration.

2.11. Mucoadhesivity test of cationic derivatives of GCD

10 mg of mucin was suspended in 10 ml of pH 7.4 PBS and sonicated for 5 min. Then, 0.5 ml of the obtained mixture was added to 0.5 ml of the 1 mg/ml solution of GCD and its cationic derivatives or to PBS so that the final mucin:GCD mass ratio in the solution was 1:1. The reference mucin mixture was obtained by diluting the starting suspension with PBS. To find out whether the cationic derivatives of GCD form aggregates with negatively charged mucin the DLS measurement using Nano Zetasizer was performed 30 min after mixing the solutions.

2.12. Human dermal fibroblast culture

Normal human adult dermal fibroblasts (ATCC®, PCS-201-012™) were cultured in Minimum Essential Medium Eagle (MEM, Sigma–Aldrich), supplemented with 10% fetal bovine serum (FBS; HyClone; collected in South America; triple 0.1 μm sterile filtered), MEM Non-essential Amino Acid Solution (Sigma–Aldrich), sodium pyruvate 1 mM (Sigma–Aldrich), 100 I.U./ml penicillin and 100 μg/ml streptomycin (HyClone; 0.1 μm sterile filtered) in a humidified atmosphere with 5% CO_2 at 37 °C.

2.13. Primary cultures of hippocampal neuronal cells

Hippocampal tissue for primary cultures was prepared from Swiss mouse embryos (Charles River, Germany) at 15–17 days of gestation. Animal care followed official governmental guidelines and all efforts were made to minimize the suffering and the number of animals used. Procedures were carried out in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and studies were approved by the Bioethics Commission in compliance with Polish Law (21 August 1997). The cells were cultured as previously described [14,17]. They were suspended in estrogen-free neurobasal medium supplemented with B27 and plated at a density of 2.5×10^5 cells per cm^2 on polyornithine (0.01 mg/ml)-coated multi-well plates. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2 for 7 days in vitro (DIV) prior to experimentation. The amount of astrocytes, as determined by the content of intermediate filament protein GFAP (glial fibrillary acidic protein) did not exceed 10% for all cultures [16].

2.14. Treatment and assessment of the cellular distribution of NR in hippocampal neuronal cells

Primary neuronal cell cultures were exposed to GCD-EDA/NR complex (10 and 20 μg/ml) for 24, 48 and 72 h. The complex was originally dissolved in H_2O and then further diluted in culture medium. To determine the cellular distribution of NR, hippocampal neuronal cells were grown on glass cover slips and subjected to the GCD-EDA/NR at 7 DIV. The cells were suspended in culture medium in a concentration of approximately 4.7×10^5/ml and then plated into 24-well culture plates in an amount of 1 ml of cell suspension per well (1.862 cm^2). After incubation with various concentrations of GCD-EDA/NR and for various periods, the samples were washed, mounted, and cover-slipped. They were analyzed with a A1-Si Nikon Inc. (Japan) confocal laser scanning system (LSCM) built onto a Nikon inverted microscope Ti-E using a Plan Apo 100×/1.4 Oil DIC objective. Images were acquired at a resolution of 2048 × 2048. The Al–Si system was equipped with a four-channel detection as well as LSBF imaging by diascopic detection of forward scattered excitation laser light during confocal laser scanning. The excitation for confocal microscopy was provided by a set of four diode lasers with excitation wavelengths at 405, 488, 561, and 638 nm.

2.15. Cell viability assay

The fibroblasts were seeded into 12-well plates at 4.3×10^4 cells per well (about 1.1×10^4 cells/cm^2) or in 100 mm Petri dishes at 7.33×10^5 cells (about 1.3×10^4 cells/cm^2). To obtain microscopic preparations the glass slides were placed on the bottom of Petri dishes prior to addition of medium and cells. 24 h after seeding the culture medium was replaced with the same medium containing different concentrations (up to 100 μg/mol) of cationic GCD or cationic GCD complexes dissolved in PBS (at 2 mg/ml, PBS content in media not larger than 5% v/v) or pure PBS as a control. Solutions of the complexes were centrifuged before use to remove the uncomplexed compounds. Fibroblasts were incubated at 37 °C for 74 h in the presence of the tested material. Next, the medium was removed and the cells were washed twice with PBS. The fibroblasts were fixed with 2 ml of 80% v/v methanol for 15 min at room temperature and treated for 2 min with Crystal Violet (CV) solution obtained by dissolving 5 g of CV in 200 ml of methanol and 800 ml of water. Unbound CV was removed by flushing with water. After drying of the surface of the wells each of them was filled with 2 ml of the extraction solution (obtained by dissolving 13.22 g of citric acid and 10.88 g of sodium citrate with 500 ml with distilled water to which 500 ml of methanol was subsequently added) for 30 min and then the solution absorbance was measured at 540 nm. The maximum concentration of the complexes used in further biological experiments was equal to the value of the concentration of the corresponding cationic GCD that caused a 10% decrease in the cell viability.

2.16. Total GAGs assay in fibroblasts

For total GAGs assay the fibroblasts were fixed with 4% formamide in PBS for 1 h at 4 °C. The fixative was removed and cells were washed twice with 0.1 M HCl and stained using 0.5% solution of Alcian Blue 8GX (AB) in 0.1 M HCl for 5 h. Then, the unbound AB was removed by flushing with water and the surface of the wells was dried. The wells were filled with 0.5 ml of the extraction solution containing 0.5% w/v SDS and 50 μg/ml proteinase K in PBS.

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Fibroblasts were digested for 0.5 h at 56 °C and then the absorbance of the solution was measured at 680 nm.

2.17. Fluorimetric determination of DAI extracted from fibroblasts

The fibroblasts were seeded into 12-well plates at 4.3×10^4 cells per well (about 1.1×10^4 cells/cm^2) or in 100 mm Petri dishes at 7.33×10^5 cells (about 1.3×10^4 cells/cm^2) and cultured to reach 30% confluency. Then, they were supplemented with DAI complexes in cationic GCD to in the form of a solution obtained by mixing 0.5 ml of 1.0 mg/ml solution of the respective DAI complex with a cationic GCD in PBS and 9.5 ml of media (the final concentration of the DAI complex was 50 μg/ml). The cells were cultured for another 3 DIV. Then, the medium was removed and the dishes containing the cells were washed twice with 3 ml of PBS. Next, extraction was carried out by adding 2 ml of methanol for 10 min to previously dried Petri dish containing cultured fibroblasts followed by gentle agitation. Fluorescence of DAI in methanol was measured using excitation wavelength of 305 nm.

2.18. Microscopic imaging of NR in fibroblasts

The fibroblasts were seeded into 12-well plates at 4.3×10^4 cells per well (about 1.1×10^4 cells/cm^2) or in 100 mm Petri dishes at 7.33×10^5 cells (about 1.3×10^4 cells/cm^2) and cultured to reach 30% confluency. Then, they were supplemented with NR complexes in cationic GCD in the form of a solution obtained by mixing 0.5 ml of 1.0 mg/ml solution of the respective NR complex with a cationic GCD in PBS and 9.5 ml of media (the final concentration of the NR complex was 50 μg/ml) and the cells were cultured for another 3 DIV. Saturated solution of uncomplexed NR used as a reference was obtained by suspending NR at 1 mg/ml in PBS, vortexing for 15 min and removing undissolved NR by centrifugation at 4000 rpm (1696g) for 2 min. The NR solution was then added to the cultured cells (30% confluent) at 5% v/v.

3. Results and discussion

3.1. Characterization of the cationic GCD derivatives

Two cationic γ-cyclodextrin (GCD) derivatives were obtained by substitution of the hydroxyl groups of GCD with two different cationic compounds (Fig. 1). The first of them was glycidyltrimethylammonium chloride (GTMAC), an epoxy ammonium compound, which can be used for the pH-independent cationization of the polymers by reacting with their amino [35,12], hydroxyl [46,10,37], or carboxyl groups [30]. The other compound was ethylenediamine (EDA), which may be used as a pH-dependent cationizer of proteins [39], polysaccharides [49], synthetic polymers [27,47,50], lipids [28], as a linker for subsequent attachment of GTMAC [34], or to functionalize cyclodextrin cores for the synthesis of star-shaped polymer [7]. The products of these reactions are referred to in the text as GCD-GTMAC and GCD-EDA, respectively. To the best of our knowledge, none of these compounds has been studied as a cationic vehicle for the delivery of sparingly water soluble drugs.

Modification of GCD with GTMAC and EDA was confirmed using FT-IR and ^{1}H NMR spectroscopies and elemental analysis (see Figs. S1–S4 and Table S1 in Supplementary Information).

In the FTIR spectrum of GCD-GTMAC a new band appeared at 1480 cm^{-1} characteristic of asymmetric angular bending vibration of CH_3 groups bound to the quaternary nitrogen atom of GTMAC. In addition, in the ^{1}H NMR spectrum a signal appeared at 3.1 ppm which is characteristic of methyl protons and a signal at 4.4 ppm characteristic of the hydroxyl proton in the attached GTMAC group.

In the FTIR spectrum of GCD-EDA new bands at 2800 cm^{-1} from CH—NH stretching vibration, at 1500 cm^{-1} from N—H deformation vibration, and at 1360 cm^{-1} from C—N vibration are visible. In the GCD-Ts spectrum a 1200 cm^{-1} band from S=O stretch and a 1615 cm^{-1} band from skeletal aromatic ring vibration appeared. In the ^{1}H NMR spectrum of GCD-EDA new signals appeared at 2.6–2.8 ppm, originating from protons bound to the C6 glucose carbon to which EDA moiety is attached [32], and signals between 3.5 and 4.0 from methylene protons in EDA.

The successful cationization of GCD was also confirmed with elemental analysis indicating the presence of nitrogen in both cationic GCD derivatives (see Table S1 in Supplementary Information). The results of the elemental analysis were used to calculate the average degree of substitution (DS) of GCD with GTMAC or EDA, defined as the number of GTMAC or EDA groups attached to one glucose unit of GCD. The average values of DS for GCD-GTMAC and GCD-EDA were found to be 0.99 and 0.68, respectively, which means that a GCD molecule was substituted with 7.9 GTMAC groups and 5.4 EDA groups on the average, respectively.

3.2. Interaction of cationized GCD with anionic biopolymers

We have studied whether the cationic GCD derivatives obtained are capable of interacting with negatively charged biomacromolecules. For that purpose we have selected unfractionated heparin (UFH), the glycosaminoglycan (GAG) with the highest negative charge density and a strong blood anticoagulant [11], and mucin,

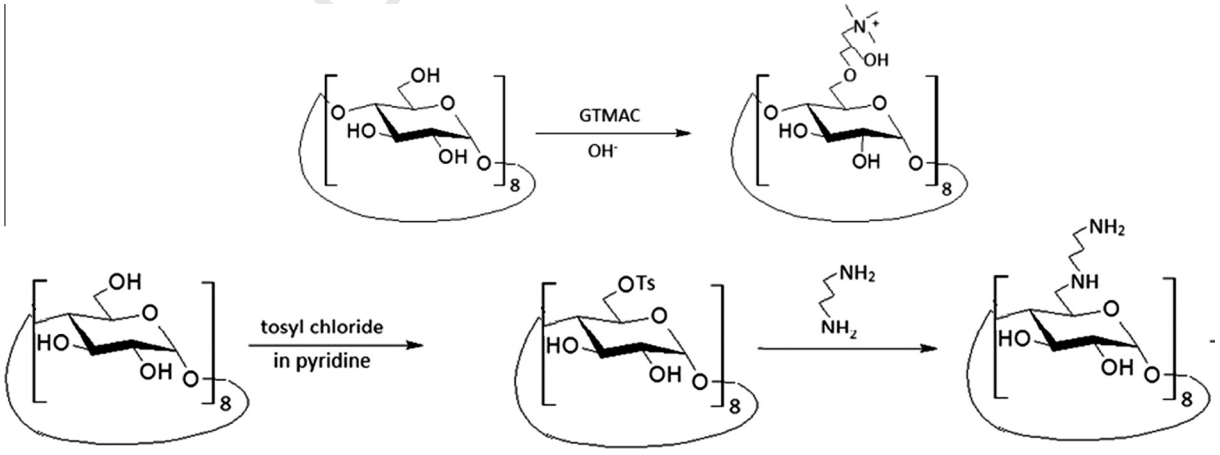

Fig. 1. Synthesis of cationic GCD derivatives.

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a heavily glycosylated gel-forming protein which is responsible for intestinal homeostasis and defense against microorganisms [9].

We have found strong interaction between both cationic GCD derivatives and UFH resulting in the formation of micrometer-sized aggregates as found from the dynamic light scattering (DLS) measurement (Fig. 2a).

This interaction led to the reduction in the concentration of free (uncomplexed) UFH in the solution (see Fig. S5 in Supplementary Information) suggesting that cationic GCD derivatives may inhibit anticoagulative activity of UFH, as we have found for several synthetic polycations [19] and several cationically modified natural polymers [24,18]. We have confirmed (data not shown) that cationic GCDs indeed inhibited anticoagulative activity of UFH. These studies are out of the scope of the present paper and will be reported elsewhere.

The interaction of the obtained compounds with mucin was also studied to test their mucoadhesivity. This was done by measuring the change in the size of the objects formed by mucin upon addition of GCD or its cationic derivative using the DLS technique (Fig. 2b). It was found that the size of negatively charged mucin particles increases significantly upon addition of both cationic derivatives of GCD apparently due to the electrostatic attraction (Table 1). It was also found that unmodified GCD did not cause any changes in the size of mucin particles in the solution confirming that the mucoadhesivity of GCD derivatives is due to their cationic character. These results indicate that both cationic GCD derivatives are mucoadhesive and may be used to obtain mucosal delivery systems.

3.3. Investigation of DAI complexation by cationic GCDs with circular dichroism and infrared spectroscopy

In the next step it was studied whether the cationic GCD derivatives are able to form inclusion complexes with DAI. To answer this question circular dichroism spectra of DAI in the presence of GCD derivatives were measured (Figs. S6 and S7 in Supplementary Information). The spectra show the presence of positive induced circular dichroism (ICD) band corresponding to the 262 nm absorption band of DAI. This is a direct evidence that DAI enters the chiral cavities of GCD-GTMAC and GCD-EDA forming inclusion complexes.

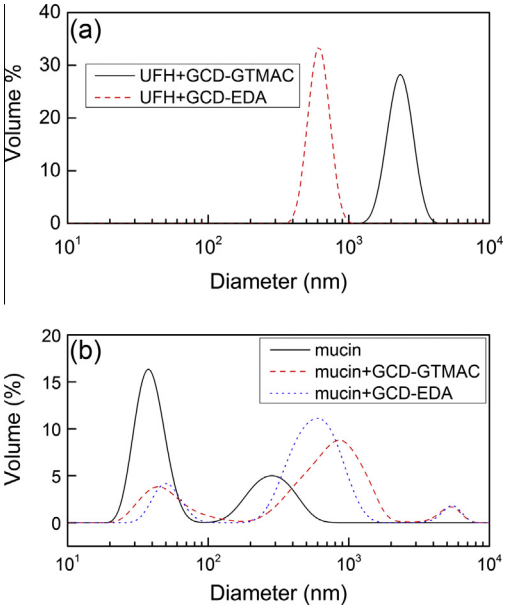

Fig. 2. (a) The size of the cationic GCD-UFH complexes found from DLS (c_{UFH} = 0.2 - mg/ml, $c_{GCD\text{-}GTMAC}$ = 0.7 mg/ml, $c_{GCD\text{-}EDA}$ = 0.5 mg/ml, room temperature). The samples were shaken for 10 min before the measurement. The light scattering for pure cationic GCD derivatives and UFH was too weak to give meaningful signals; (b) The size of objects formed by mucin and the size of its complexes with cationic GCDs (c_{mucin}, $c_{GCD\text{-}GTMAC}$, $c_{GCD\text{-}EDA}$ were 0.5 mg/ml). The samples were shaken for 30 min before the measurement. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
The size of objects formed by mucin and its aggregates with cationic GCD derivatives.

System	Size of the objects ± SD (nm)
Mucin	285.1 ± 8.1
Mucin + GCD-GTMAC	525.1 ± 12.8
Mucin + GCD-EDA	566.3 ± 8.6

3.4. IR spectra of complexes and physical mixtures

The formation of DAI complexes was further studied using infrared spectroscopy. It was reported that complexation of a compound by a cyclodextrin may be manifested as the difference between the IR spectrum of a physical mixture of the complex components and the spectrum of the complex [4]. The comparison of the spectra of DAI complexes, the corresponding physical mixtures and pure components is shown in Supplementary Information (Figs. S8 and S9). In the case of physical mixtures the presented spectra are a superposition of the spectra of their components, while the spectra of the complexes exhibit a much greater resemblance to the spectrum of the cyclodextrin. This observation is another confirmation of the formation of inclusive complexes of DAI.

3.5. Solubilization of daidzein with GCD and its cationic derivatives

The effect of increasing concentrations of both cationic GCD derivatives on DAI solubility was studied and compared to that of parent GCD (Fig. 3). It was found that GCD does not appreciably increase concentration of DAI. For GCD-GTMAC the solubilization effect was noticeable although rather small while GCD-EDA strongly complexed DAI and increased its concentration in an aqueous solution. Similar behavior was found for β-cyclodextrin which barely solubilized DAI, in contrast to its derivatives, i.e., hydroxypropyl-β-cyclodextrin (HP-β-CD) and random methyl-β-cyclodextrin (RAMEB), which significantly enhanced DAI and genistein solubility [6]. Since the molar concentration of solubilized DAI was higher than the molar concentration of GCD-EDA, it could be concluded that one GCD-ECD molecule may complex more than one DAI molecule, on the average.

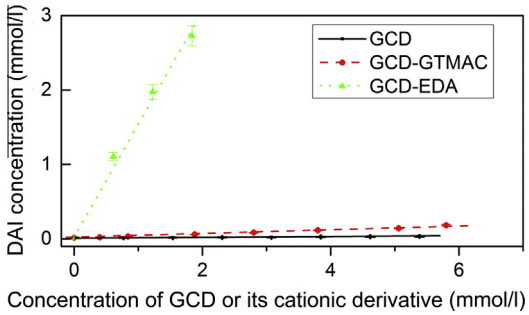

Fig. 3. The relationship between the concentration of dissolved DAI and the concentration of GCD and its cationic derivatives in the aqueous solution. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Based on the data shown in Fig. 3 the values of the binding constant, *K*, could be calculated for GCD and its both cationic derivatives (see Table 2 and the Supplementary Information). Also, the complexation efficiency (CE) was calculated for GCD/DAI and GCD-GTMAC/DAI complexes assuming 1:1 stoichiometry which is considered a more accurate measure of the solubilizing effect of cyclodextrins [23] than the binding constant. The differences between CE values for different DAI complexes are even larger than those between their *K* values. It should be, however, kept in mind that these are the average values since both GCD derivatives are a mixture of molecules with different DS.

The values of *K* were found to be higher for GCD-GTMAC and GCD-EDA than for the parent GCD by one and more than two orders of magnitude, respectively. The value of *K* for GCD-EDA is close to the limiting value of 10^5 required for the CD to have an influence on the drug pharmacokinetics after parenteral administration [36]. Stronger complexation of DAI by the GCD derivatives than by parent GCD seems to originate from the possibility of the hydrogen bond formation between DAI and EDA or GTMAC moieties in the substituted GCD [48]. Also, the *K* and CE values of GCD-GTMAC much lower than those of GCD-EDA may be due to the fact that the former has much higher DS (almost each glucose unit in GCD is substituted on the average) and the GTMAC groups are more bulky than EDA moieties so the complex formation with DAI may be sterically hindered by the GTMAC groups. The strong DAI solubilization effect found for GCD-EDA makes it a promising vehicle for the delivery of DAI. It may be also expected that cationic modification would prevent GCD derivatives studied from aggregation which is a characteristic yet unfavorable property of the parent GCD [22].

3.6. Effect of cationic GCDs and their complexes with DAI on cell viability

The effect of cationic cyclodextrins on the viability of fibroblasts was studied with a method based on the use of Crystal Violet staining (CVS) test. The concentration causing a statistically significant decrease in cell viability by 10% (Fig. 4) for both GCD-GTMAC and GCD-GTMAC/DAI was 100 μg/ml and was used as the maximum concentration in further biological studies. For GCD-EDA the decrease in the cell viability was lower than 10% up to 100 μg/ml while for GCD-EDA/DAI the 10% viability decrease occurs for the concentrations of 80 μg/ml and higher of the complex. The difference in toxicity between GCD-GTMAC/DAI and GCD-EDA/DAI may be ascribed to much higher DAI solubilization efficiency of GCD-EDA, as found in the solubilization studies described above, on one hand, and to strong decrease in cellular GAGs level caused by DAI on the other (see below).

3.7. Studies on the Nile Red delivery in cationic GCD complexes

We have performed studies on the possibility of the application of cationic GCDs obtained as vehicles for the delivery of water-insoluble substances to the cells. To find out whether cationic GCDs enter the cells the complexes of these compounds with Nile Red (NR) were obtained. NR is poorly water soluble and it does not fluoresce in the aqueous solution while in hydrophobic media it

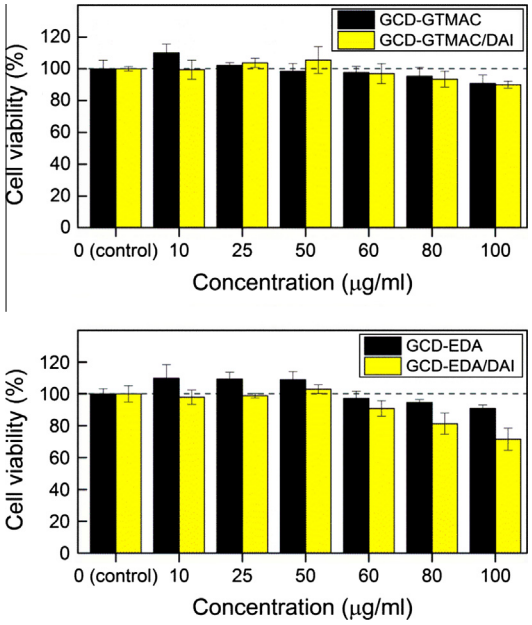

Fig. 4. Dependence of fibroblast viability on the concentration of cationic derivatives of GCD in the absence and in the presence of complexed DAI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

emits strong fluorescence. We have supplemented the medium used to culture the fibroblasts with cationic GCDs/NR complexes. A medium saturated with NR was used as a control. The confocal images of the fibroblast cultures (Fig. 5) show significant fluorescence of NR located in the cytoplasm of the fibroblasts cultured in the media containing cationic GCDs/NR complexes, while the NR fluorescence from the fibroblasts cultured in NR-saturated medium is very weak. This indicates that cationic GCDs increase the uptake of NR and may serve as efficient delivery systems for water-insoluble substances. Also, there are some differences in the images obtained for both cationic GCDs. The fluorescence of NR delivered in GCD-GTMAC is mostly concentrated in well-defined spots while for NR delivered in GCD-EDA it is generally much stronger and, except for fluorescent spots visible also for GCD-GTMAC/NR, the areas of cytoplasm showing diffuse fluorescence can be seen. This observation confirms that GCD-EDA is a better delivery vehicle than GCD-GTMAC.

Analogous experiments were performed for the murine hippocampal neuronal cells. The NR fluorescence emitted from these cells confirms the uptake of complexed NR by these cells (Fig. 6).

3.8. Studies on the DAI delivery in cationic GCD complexes

The possibility of the delivery of DAI complexed by the cationic GCD derivatives was then studied. For this purpose the fibroblasts were cultured in media containing complexed DAI or saturated with DAI (as a control). The cells were then extracted with methanol and the fluorescence spectra of the extracts were measured at λ_{ex} = 305 nm (Fig. 7). It was found that extracts from cells cultured in the presence of GCD-EDA/DAI emitted fluorescence identical to that of DAI, while those from the fibroblasts cultured in the presence of GCD-GTMAC/DAI show much weaker fluorescence.

These results confirmed the presence of DAI in the cells and indicate that DAI may be delivered to the cells by cationic GCD derivatives studied. Moreover, GCD-EDA seems to be a more efficient system for DAI delivery than GCD-GTMAC.

Table 2
Binding constants and complexation efficiency of DAI by GCD and its derivatives.

Compound	Binding constant *K*	Complexation efficiency CE
GCD	3.26×10^2	3.58×10^{-3}
GCD-GTMAC	3.20×10^3	2.88×10^{-2}
GCD-EDA	6.42×10^4	1.47×10^0

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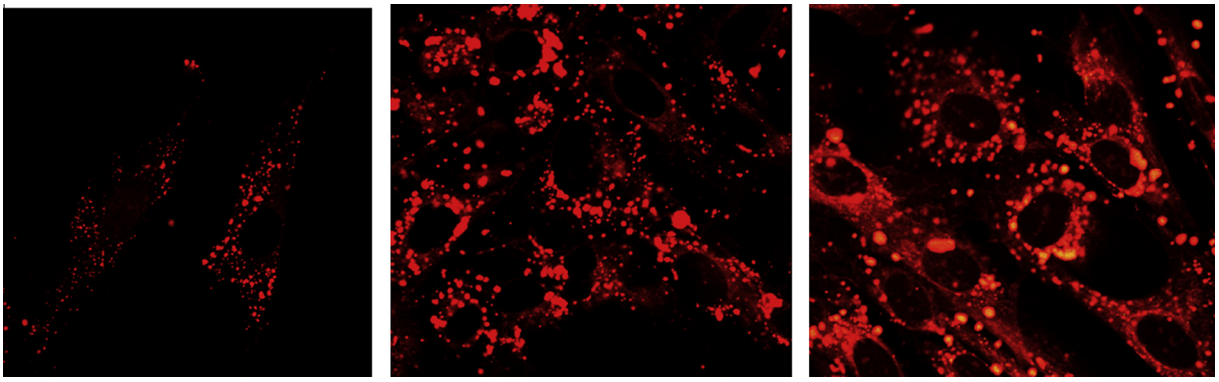

Fig. 5. Confocal microscope images of fibroblasts cultured in a medium saturated with NR (left) and containing 50 μg/ml of GCD-GTMAC/NR (middle) and GCD-EDA/NR (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

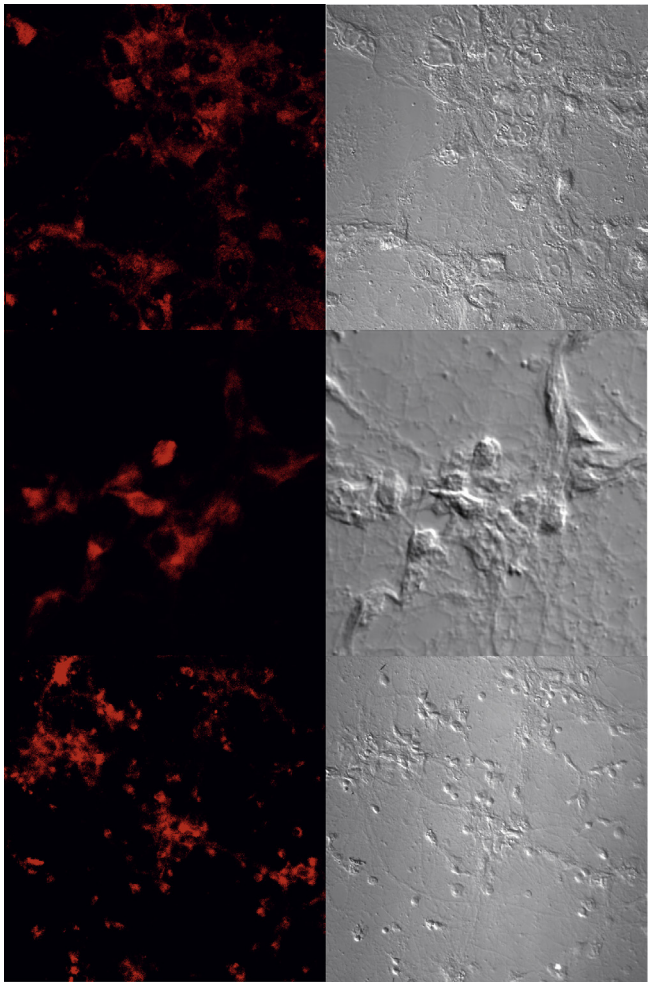

Fig. 6. NR fluorescence (left) from murine hippocampal neuronal cells (bright field, right) cultured in the medium containing GCD-EDA/NR. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.9. Effect of DAI delivered complexes on the level of total glycosaminoglycans in fibroblasts

Once the successful delivery of DAI complexes to the fibroblasts was confirmed, we have studied if it decreases the GAGs level in the fibroblasts, as could be expected based on the well-known influence of isoflavones on the GAGs biosynthesis. For this purpose fibroblasts were cultured in media containing increasing concentrations of DAI complexes with both cationic GCDs and the level of GAGs was determined using Alcian Blue 8GX staining method. The results are shown in Fig. 8.

DAI delivered in both complexes results in a decrease of total GAGs level. For DAI delivered in GCD-GTMAC/DAI the level of GAGs started to significantly decrease at the complex concentrations of 80 μg/ml and higher, while a significant decrease of GAGs starts at the concentration of GCD-EDA as low as 10 μg/ml. For 100 μg/ml of the complex which was the highest complex concentration studied, the GAGs level dropped down to 72 ± 4% and 62 ± 10% of the control value for GCD-GTMAC/DAI and GCD-EDA/DAI, respectively. Such a significant decrease of cellular GAGs could be the reason of reduced cell viability seen for high concentrations of GCD-EDA/DAI. It should be noted, however, that at the concentration range from 10 to 80 μg/ml GCD-EDA/DAI complex does not adversely influence the cell viability (see above) while it significantly decreases the GAGs level. Importantly, the decrease of cellular GAGs level can be ascribed solely to DAI since neither GCD-GTMAC nor GCD-EDA alone influenced the GAGs level. The increased uptake of complexed DAI by the fibroblasts, and particularly by the hippocampal neuronal cells, a decrease of total GAGs found after administration of complexed DAI, together with the low toxicity of the cationic GCDs make them promising delivery systems for the treatment of mucopolysaccharidoses (MPSs). To confirm this assumption further studies will be performed using MPS cells.

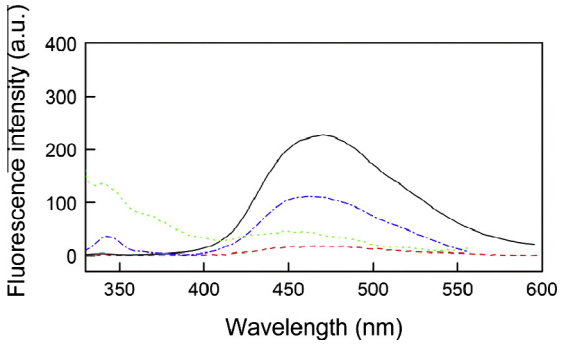

Fig. 7. The fluorescence spectra of the methanol extracts of the fibroblasts cultured in the presence of 50 μg/ml GCD-GTMAC/DAI (dot) and GCD-EDA (dash dot) and the fluorescence DAI spectra in methanol (solid, 0.098 mmol/l) and in water (dash, saturated solution) (λ_{ex} = 305 nm). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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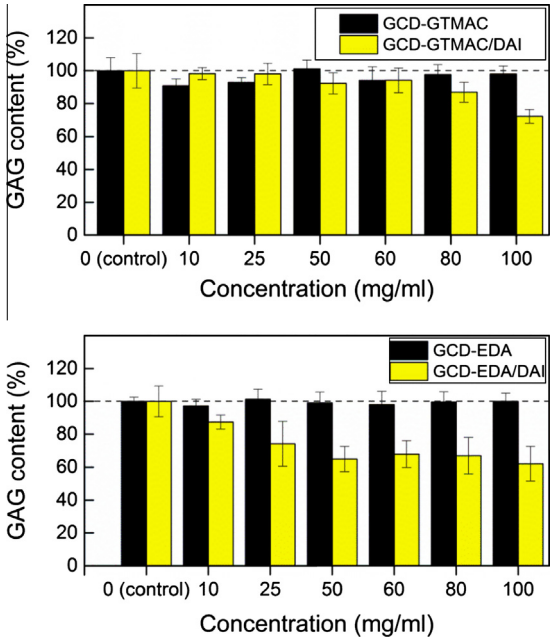

Fig. 8. Dependence of total GAGs level in fibroblasts on the concentration of cationic derivatives of GCD in the absence and in the presence of complexed DAI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusions

The cationic derivatives of γ-cyclodextrin were found to form inclusion complexes with daidzein, an isoflavone which decreases the pathological glycosaminoglycan storage in mucopolysaccharidoses and therefore it is considered as a potential drug in the treatment of these diseases. We found enhanced absorption of daidzein complexed with cationic obtained cyclodextrins by human dermal fibroblasts and murine hippocampal neuronal cells. The level of cellular glycosaminoglycans in fibroblasts could be significantly decreased in the fibroblasts upon administration of complexed daidzein.

Conflict of interest

Kamil Kamiński, Monika Kujdowicz, Małgorzata Kajta, Maria Nowakowska, and Krzysztof Szczubiałka declare that they have no conflict of interest.

Animal rights

All institutional and national guidelines for the care and use of laboratory animals were followed.

Contributions of individual authors

Dr. Kamil Kamiński and Monika Kujdowicz, MD, performed the syntheses, physicochemical measurements and performed in vitro studies in human adult dermal fibroblasts.

Prof. Małgorzata Kajta performed experiments with murine hippocampal nervous cells.

Prof. Maria Nowakowska and Prof. Krzysztof Szczubiałka planned the experiments, analyzed the results and wrote the manuscript.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2015.02.002.

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