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Synthesis and Characterization of a Colloidal Novel Folate- β-CD Conjugate for Targeted Drug Delivery

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Abstract

Abstract A novel folic acid–b-cyclodextrin (b-CD) conjugate was synthesized and preliminarily characterized by $_1$H NMR, ESI-MS, and MALDI-MS. $_1$H NMR shows the presence of a- and c-conjugates which are generated by b-CD linkage in turn with both carboxylic functions of folic acid. Moreover ROESY evidences supramolecular interactions between the benzene ring of the folic acid and the b-CD cavity. DOSY suggests that ethylenediamine derived b-CD–folic acid forms a colloidal dispersion difficult to purify from free folic acid. An analysis of self-diffusion coefficient (D_s) of the three species (a-, c-conjugates, and free folic acid) and relaxation times (T_1 and T_2) is reported to tentatively explain the colloidal behaviour of the new species in an aqueous solution.

Keywords Cyclodextrin, Folic acid Conjugates, Dynamic NMR, Colloids

Introduction

One of the most important goals of pharmaceutical science is directing the pharmacological activity of the drug to the required site of action. Drug delivery systems (DDSs) are molecular tools which target a specific receptor without undesired interactions at other sites [1, 2]. These molecular systems can also offer controlled drug release. Advanced DDSs include a targeting moiety and supplementary active ingredients including the carrier and drug [3]. DDSs are made from a variety of organic and inorganic compounds such as polymers, lipids (liposomes, nanoemulsions, and solid-lipid nanoparticles), self-assembling amphiphilic molecules, dendrimers, and inorganic nanocrystals [4]. Cyclodextrins (CDs) improve the solubilization and stabilization of drugs and they can be potent drug carriers for immediate and delayed delivery [5]. CDs and their derivatives, especially colloidal amphiphilic CDs, also increase drug permeability by direct action on mucosal membranes and enhance drug absorption and/or bioavailability [6, 7]. Folic acid (FA) has been used as a cancer targeting agent and is recognized by tumour cells which over-express folate receptors [8]. FA receptors can be direct targets, involving antagonist drugs, or indirect targeting tools for delivery involving competitive drugs. This explains the several strategies used for folate conjugation. FA has also been conjugated to polyethyleneglycol (PEG) to successfully deliver a wide variety of compounds including chemotherapeutic agents, oligonucleotides, photosensitizers, polymers and dendrimers [9]. The synthesis of a b-cyclodextrin–polyethyleneglycol–folic acid conjugate (CD-PEG-FA) has been reported by Caliceti et al. CD-PEG-FA was obtained from the reaction between CD-PEG-NH_2 and FA [10]. The

conjugate with FA was proposed as an active tumour targeting molecule [11]. Another conjugate folate–PEG–folate-graft-polyethyleneimine has also been reported and is described in the literature as a potential gene carrier [12]. Recently synthetic procedures for the production of nanoparticles decorated with FA conjugates have been reported [13, 14]. This work aims to develop new vehicles as DDSs for cancer therapy applications. The synthetic strategy followed is based on the production of a new 6-[(2-aminoethyl) amino]-6-deoxy-b-cyclodextrin–folic acid (CDEn- FA) which involves reaction between an ethylenediamine modified b-CD and FA. The recognition properties of permodified folate-b-CD conjugates were evaluated with cancer cell lines overexpressing the folate receptor [15]. In this paper we report the synthesis of CDEn-FA where FA is directly linked to 6-[(2-aminoethyl)amino]-6-deoxyb- cyclodextrin (CDEn) in an attempt to eliminate the polydispersity observed in CDs modified with polymers such as PEG. In this way we aim to obtain a molecular system with a short spacer which presents a controlled number of binding sites (En) prompt to coordinate metal ions and a receptor targeting group (FA). The novel folic acid–CD conjugate was characterized using a range of techniques including NMR, MALDI-MS, and ESI-MS. The polidispersity due to the PEG was eliminated. However the novel CD conjugate may contain free FA which is difficult to remove. The system can form a colloidal dispersion which was characterized by DOSY. Supramolecular interactions between FA moieties and the CD cavity were revealed by ROESY to get insight on the structure of CDEn-FA in water. Finally preliminary analytical HPLC experiments were performed to separate the two observed isomeric conjugates and free FA.

Materials and methods

Reagents were obtained from Sigma-Aldrich unless otherwise indicated. Pyridine and DMSO were anhydrous from sealed bottles under N_2 atmosphere. TLC was carried out on Merck Silicagel 60 analytical plates with the specified solvent system. CD derivatives were detected by UV light or by dipping in 5% H_2SO_4 in EtOH and heating. NMR spectra were recorded on a Bruker Avance spectrometer. ^{1}H NMR spectra were recorded at 700 MHz and ^{13}C NMR spectra at 175 MHz. Samples were dissolved in D_2O and the ^{1}H NMR reference peak was at 4.76 ppm for HOD. The sample temperature was kept at 298 K and controlled by a cold N_2 flow and a heating element, calibrated against the standard CH_3OH reference (4% CH_3OH in CD_3OD) with an accuracy of 0.2 K. The self-diffusion coefficient was measured by means of Pulse Field Gradient Stimulated Echo (PGSTE) technique [16]. A gradient pulse of duration d = 4 ms and a diffusion time D = 100 ms were used to cover the entire dynamic range of the echo attenuation varying the values of the field gradients from 2 to 50 G cm^{-1}. Furthermore the spin–spin (T_2) and spin– lattice (T_1) relaxation times were recorded by means of the Carr–Purcell–Meiboom–Gill (CPMG) and Inverse Recovery Pulse sequences respectively. A Shimadzu HPLC system was used, equipped with a SCL-10A VP controller system, a Shimadzu LC-10 AD VP solvent delivery module, and a SPD-M10A VP UV/Vis photodiode array detector (PDA). The stationary phase was a Supelco column RP C-18 (4.6 9 25 mm) with a mobile phase of phosphate buffer solution (PBS) (10 mM, pH 2.27): CH_3CN 8:92 v/v. Measurements were carried out with a flow rate 0.5 mL/min at room temperature (25 _C). MALDI-TOF analyses were performed on a Perspetive (Framingham, MA) Voyager STR instrument equipped with delayed extraction technology. ESI-MS was carried out on a Thermo LXQ linear trap, with potential full scan at nuzzle of 4.5 kV, flow 5 lL/min and sheath gas N_2 at 20 a.u. (arbitrary unit). The system can be used to determine molecular masses in the range 50– 100000 Da, with an accuracy of 20 ppm. The samples were introduced into the mass spectrometer in solution and ionized at atmospheric pressure.

Synthesis of CDEn-FA conjugate

CDEn was prepared as previously described [17, 18]. CDEn (0.2000 g, 0.15 mmol) was dissolved in pyridine (15 mL). FA (0.0330 g, 0.07 mmol) and N-hydroxysuccinimide (NHS) (0.0350 g, 0.31 mmol) were dissolved in DMSO (10 mL) and added to the CDEn solution. Finally, Dicyclohexylcarbodiimide (DCC) (0.0630 g, 0.31 mmol) was also added. The solution was stirred in the dark overnight under nitrogen at 30 _C. The solution was then warmed to 45 _C for 2 h and was added to $(CH_3)_2CO$ (50 mL) at -20 _C. TLC (5:1:3:2 PrOH/Ac_2O/H_2O/NH_3; R_f = 0.53) was used to monitor the reactions. The produced precipitate was left to stand overnight. About 0.1 g of crude product was recovered by filtration and washed with $(CH_3)_2CO$, CH_3CN, and Et_2O. The crude product was purified by column chromatography (15 9 150 mm) using CM Sephadex C25 40-125l (Sigma) with gradient elution by 070.2 M NH_4HCO_3 (total volume 300 mL) and by Sephadex G-25 20–80 l (20 9 300 mm) by using H_2O as eluent (150 mL). The resulting solid was dried in vacuo (Yield: 5%).

^{1}H NMR (D_2O) d (ppm): 8.65 (s, H-31), 7.70 (d, H-19,H-23), 6.67 (d, H-20, H-22), 4.97 (br, H-1$_{CD}$), 4.54 (m,H-25), 3.94 (s, H-14), 3.77 (br, H-6$_{CD}$), 3.70 (br, H-3$_{CD}$), 3.60 (br, H-5$_{CD}$), 3.53 (br, H-2$_{CD}$, H-4$_{CD}$), 3.45 (br, H-6$_{0CD}$), 2.65 (br, H-8), 2.23 (br, H-12), 2.08 (br, H-7), 1.87 (br, H-13).
ESI-MS: m/z = 1600.7 [M ? H]$^?$, 1622.7 [M ? Na]$^?$,
MALDI-MS m/z: 1602.6 [M ? H] $^?$, 1624.7 [M ? Na] $^?$.
HPLC (UV–Vis detector): t_R 4.40 min (198, 275, and 365 nm), 5.00 min; (198, 280 and 365 nm).

Results and discussions

The CDEn-FA conjugate was prepared by reaction of FA with CDEn in the presence of NHS and DCC (see Scheme 1). Despite many purification steps being carried out, free FA was still present. These findings have not permitted a rigorous integration of the peaks in the ^{1}H NMR spectrum. There are no reports in the literature of the synthesis of CDEn-FA. Mass analysis confirms the presence of the CDEn-FA conjugate showing peaks which were assigned to the molecular peak plus proton and sodium ion. The ^{1}H NMR spectrum of CDEn-FA was assigned for comparison with the spectrum of free FA [19] and by COSY. ^{1}H NMR shows signals in the region 8.7–6.5 ppm which can be assigned to the aromatic protons. However there are three signals for each aromatic proton. This suggest that the FA moiety is present in three different species that is FA is probably bonded to CDEn in turn with both carboxylic functions generating a- and c-conjugates, and it is also present as unmodified compound. In preliminary way, it was possible to find the conditions to purify the conjugates from the free FA by analytical HPLC and therefore to isolate the two isomers. In fact the UV–Vis spectra (not shown) in correspondence of the peaks at 4.40 and 5 min show absorption bands at 198, 280, and 365 nm which were tentatively ascribed to the absorption bands of the CD cavity, and pteridine and benzene rings of FA moieties, respectively.
Further evidence for the presence of a- and c-conjugates was found using ROESY spectroscopy, and Fig. 1 shows the obtained spectra. It was possible to assign respectively H-19a (or H-23a), H-19c (or H-23 c) and H-20a (or H-22a), H-20c (or H-22 c) by correlation of the signals belonging to the same carboxylic acid groups of both conjugates. It was also possible to show that the benzene group of the FA can interact with the CD cavity by looking at the correlation of the peaks assigned to H-19 (or H-23) and H-20 (or H-22) of FA (6.59 and 7.51 ppm respectively) with the peaks assigned to the hydrogens of the CD cavity at 3.5–4 ppm. However deep inclusion of the FA group within the cavity can be excluded since no cross-peak between the proton of pteridine ring (H-31 at 8.65 ppm) and the CD cavity was detected. These results point out that a supramolecular interaction between FA of CDEn-FA and the CD cavity of another close molecule could be possible and could be responsible for the clustering process of conjugate in aqueous solution. The conformation and structural properties of the new conjugate in water are presently under investigation. A direct confirmation that CDEn-FA forms a colloidal dispersion and that there are three different types of FA arrangements in the colloidal environment is also given by a simple inspection of the NMR data given in Table 1. In fact, the measured values of the self-diffusion coefficient for the FA in aqueous solution is 3.58 9 10^{-10} m^2/s, which is very close to the value of the aromatic proton labeled ‘‘FA’’ within our system. On the other hand, the measured value of the self-diffusion coefficient of free b-CD in an aqueous solution (7.5 mM) is 2.74 9 10^{-10} m^2/s which is comparable with that of the b-CD protons in the studied system which, in turn, is similar (within the experimental error) to those of the FA protons labeled a and c. By looking at the order of magnitude of the self-diffusion coefficient it is possible to infer that the size of the diffusing particles is on the nanometric scale.
It is possible to draw the same conclusion also by exploring the values of the NMR relaxation times (T_1 and T_2) which are shorter for the pure FA protons compared to the b-CD bonded protons. Thus, with respect to T_1 values, the interactions among the FA molecules not covalently bonded to the CD and their environment are stronger than those that take place between the FA conjugate to the CD and their environment. Then again, with respect to T_2 values, the interactions among free FA molecules are stronger with respect to those which take place between the FA moieties of the conjugates [20]. This could explain the difficulty in purifying the sample. This investigation could be useful from the perspective of applications of colloidal assemblies of CD modified with targeting groups encapsulating guests for selective drug delivery. In this direction our studies are advancing.

Conclusion

A novel b-CD conjugate was synthesized by a condensation reaction between CDEn and FA. ^{1}H NMR spectra suggest the presence of two isomers (a and c) which are formed by reaction of the two FA carboxylic functions (C-11 and C-15, respectively). ROESY shows the interaction between the benzene ring of the grafted folic residue and the CD cavity and seems to exclude the interaction between the macrocycle and the terminal pteridine ring of the targeting unit. This interaction between different molecules of CDEn-FA is probably responsible for the presence of colloidal clusters in water. A close inspection of DOSY parameters (D_s), and T_1 and T_2 again supports the suggestion that there are three different types of FA arrangements in the colloidal environments (two isomeric conjugates and free FA). These studies show that free FA is difficult to remove, and this fact is probably due to the stronger interactions among free FA molecules with respect to those which take place among the FA moieties of the conjugates.

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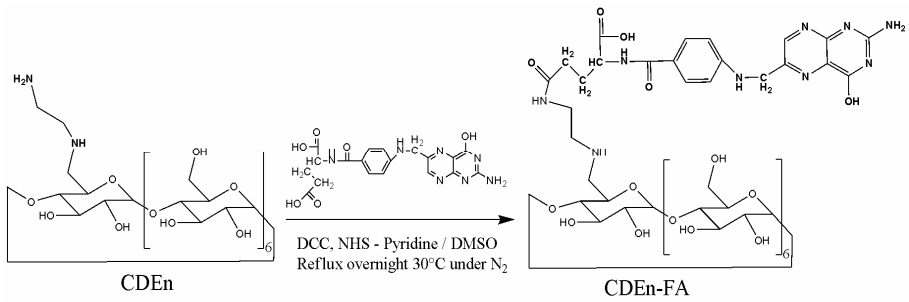

Scheme 1: Synthesis of CDEn- FA conjugate obtained by reaction of CDEn and FA.

Peak position (ppm)	$D_s.E^{-10}$ (m^2/s)	T_2 (s)	T_1 (s)
8.67 (H-31 FA)	3.45	0.09	1.07
8.63 (H-31α)	2.55	0.2	1.82
8.56 (H-31γ)	2.64	0.23	1.81
7.7 (H-19 α, H–23 α)	2.61	0.17	1.29
7.6 (H-19FA)	3.57	0.12	1.06
7.53 (H-19 γ, H-23 γ)	2.64	0.24	1.4
6.73 (H-20 FA)	3.49	0.09	0.88
6.67 (H-20 α)	2.57	0.19	1.34
6.61 (H-20 γ)	2.53	0.18	1.29
3.65 ($H-5_{CD}$)	2.60	-	-

Table 1: The NMR self-diffusion coefficient (D_s), spin-spin (T_2) and spin-lattice (T_1) relaxation times values measured in the CDEn-FA system with a particular emphasis to the aromatic protons of the folic acid. The error in the estimation of the self-diffusion coefficients is of the order of 0.05x10-10 m^2/s while that regarding the relaxation times is of the order of 0.02 s.
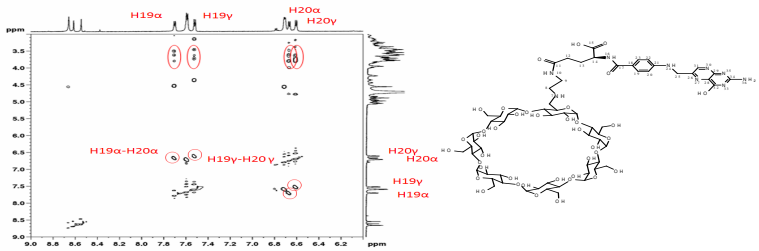
Figure 1. Left: ROESY spectrum of CDEn-Fol in D_2O, T=298 K. The cross peaks between H-19α and H-20α as well H-19γ and H-20γ are evidenced (H-23 and H-22 are omitted because they are chemically equivalent to H-19 and H-20 respectively). Right: Sketched structure of a CDEn-FA molecule. The numbering relative to the NMR characterization is also reported