

Synthesis and Physicochemical Characterization of Folate–Cyclodextrin Bioconjugate for Active Drug Delivery

Paolo Caliceti,^{*,†} Stefano Salmaso,[†] Alessandra Semenzato,[†] Tommaso Carofiglio,[‡] Roberto Fornasier,[‡] Maurizio Fermeleglia,[§] Marco Ferrone,[§] and Sabrina Pricl[§]

Department of Pharmaceutical Sciences, University of Padua, Via F. Marzolo 5, 35131 Padua, Italy, Department of Organic Chemistry, University of Padua, Via F. Marzolo 3, 35131 Padua, Italy, and Computer-aided Systems Laboratory, Department of Chemical Engineering, University of Trieste, Piazzale Europa 1, 34127 Trieste, Italy. Received May 14, 2003; Revised Manuscript Received June 18, 2003

β -Cyclodextrin–poly(ethylene glycol)–folic acid conjugate (CD-PEG-FA) was synthesized according to a two-step procedure: (1) synthesis of CD-PEG-NH₂ by reaction of monotosyl-activated β -cyclodextrin with excess of 700 Da diamino-PEG; (2) synthesis of CD-PEG-FA by reaction of CD-PEG-NH₂ with succinimidyl ester-activated folic acid. The CD-PEG-NH₂ intermediate was purified by precipitation in acetone, and the CD-PEG-FA by gel permeation and C-18 reversed-phase chromatography. Both CD-PEG-NH₂ and CD-PEG-FA were analyzed by mass spectrometry, ¹H NMR, and UV–vis spectroscopy. All analytical methods confirmed the theoretical composition of the conjugates: the CD-PEG-NH₂ intermediate was composed of CD and PEG in the molar ratio of 1:1, and the CD-PEG-FA was composed of β -cyclodextrin, PEG, and folic acid in the molar ratio of 1:1:1. The CD-PEG-FA conjugate was highly soluble in buffer (>42 mM) as compared to the unmodified β -cyclodextrin (16.3 mM). Phase solubility diagrams of β -estradiol revealed that drug solubility increases from 11 μ M in buffer to 600 μ M in the presence of β -cyclodextrins and 5900 μ M with CD-PEG-FA. However, the affinity of β -estradiol for β -cyclodextrins decreased about 4 times with PEG and folic acid conjugation. Stability studies carried out using chlorambucil confirmed that the conjugate partially prevents drug degradation in buffer, although this effect was considerably lower than that obtained with β -cyclodextrin. Computer modeling studies showed that the folic acid linked to the β -cyclodextrins through a PEG spacer could partially interact with the cyclodextrin cavity. Finally, CD-PEG-FA displayed reduced hemolytic effect as compared to unmodified β -cyclodextrin.

INTRODUCTION

Recent advances in tumor therapy demonstrate that successful anticancer strategies can be developed by employing proper carrier systems able to deliver probes, drugs, or genes to tumor targets. Many efforts are in progress to develop active drug targeting systems, which on one hand allow for specific drug delivery into the disease site and, on the other, limit the toxicological drawbacks due to the broad and nonspecific disposition of the therapeutic agents.

Folic acid is a small vitamin, which interacts specifically with the folate binding protein (FBP)¹ located in the caveole-like invaginations on the cell surface receptor (1, 2). Upon receptor interaction, the folate acid–FBP complex is taken up by cells and moves through the many organelles involved in endocytotic trafficking, providing for cytosolic deposition (2). The folic acid receptor is

overexpressed by many types of tumor cells, including ovarian, endometrial, colorectal, breast, lung, renal, neuroendocrine carcinomas, and brain metastases (3).

In virtue of its ability to be taken up by folate receptor overexpressing tumor cells, folic acid has been widely investigated as targeting molecule for active anticancer drug delivery. Proper synthesis procedures have been pointed out to link folic acid to drug carriers to produce targeting drug delivery systems. In particular, it was demonstrated that the glutamate γ -carboxyl group activation does not provoke significant loss of folic acid affinity for the receptor. Among the folate-based carriers described in the literature are radionuclide deferoxamine–folate complexes for radiopharmaceutical imaging, liposome–folate-encapsulated drugs; liposome–folate-encapsulated polylysine–DNA, and cytotoxin–folate conjugates (4–7).

Although successful results have been published, the therapeutic application of the folate conjugates is often limited by their large size. Indeed, it was established that, even though large molecules undergo passive accumulation into solid tumors by enhanced permeation and retention effect (EPR), the intratumor overpressure limits their penetration into the neoplastic mass (8, 9). Therefore, it seems rational to produce low molecular weight conjugates, which can easily reach the tumor site and be taken up actively by the tumor cells where the drug can be released.

Cyclodextrins are carbohydrate macrocycles which, by virtue of their ability to form molecular inclusion com-

* To whom correspondence should be addressed. E-mail: paolo.caliceti@unipd.it. Phone: +39 049 8275695. Fax: +39 049 8275366.

[†] Department of Pharmaceutical Sciences, University of Padua.

[‡] Department of Organic Chemistry, University of Padua.

[§] University of Trieste.

¹ Abbreviations: CD, cyclodextrins; PEG, poly(ethylene glycol); FA, folic acid; CD-PEG-NH₂, β -cyclodextrin–poly(ethylene glycol)–NH₂ bioconjugate; CD-PEG-FA, β -cyclodextrin–poly(ethylene glycol)–folic acid bioconjugate; FBP, folate binding protein; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DMSO, dimethyl sulfoxide; TEA, triethylamine.

plexes with a wide range of hydrophobic molecules, are interesting for exploitation as a new class of conjugates for drug delivery (10, 11). Actually, cyclodextrins have been investigated to optimize the solubility, stability, and bioavailability of many drugs (12–17). Nevertheless, the parenteral administration of natural cyclodextrins, namely β -cyclodextrins, causes undesirable toxic effects such as hemolysis (18). To overcome these problems, semisynthetic cyclodextrins obtained by hydroxyl substitution with hydroxy-alkyl or sulfo-alkyl functions have been prepared and investigated for systemic administration (11).

Recently, cyclodextrins have been modified by conjugation of water-soluble polymers to obtain derivatives with peculiar biopharmaceutical properties (11, 19).

Aimed at exploiting cyclodextrin bioconjugates for active drug targeting, a synthetic strategy has been investigated to produce a derivative where β -cyclodextrins are linked to folic acid through a poly(ethylene glycol) spacer arm. The goal of this paper is to report a methodology for synthesis of cyclodextrin-PEG-folate derivatives, which possess inclusion capacity of cyclodextrin, high solubility and biocompatibility of PEG, and targetable properties of folic acid for site-directed anti-cancer therapy.

MATERIALS AND METHODS

Folic acid, dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), Jeffamine ED-600 (700 Da diamino-PEG), and β -cyclodextrin were purchased from Fluka Chemika (Buchs, Switzerland). Sephadex G25 superfine resin was obtained from Pharmacia Biotech AB (Uppsala, Sweden). Chlorambucil, β -estradiol, and all other reagents were supplied by Sigma (St. Louis, MO).

Analytical thin-layer chromatography (TLC) was carried out on glass sheets coated with silica gel (Merck F-254, Merck, Darmstadt, Germany). The TLC spots were visualized with the following indicators or methods: (1) tosylated-cyclodextrin and tosyl groups, UV (254 nm); (2) carbohydrates, 9.2 mL of *p*-anisaldehyde in 338 mL of 95% ethanol, 3.75 mL of glacial acetic acid, and 12.5 mL of concentrated sulfuric acid (10–20 min at 100–150 °C becomes blue to black in the presence of cyclodextrins); (3) primary aliphatic amines, a 0.3% ninhydrin in 1-butanol containing 3% acetic acid (10 min at 125–150 °C).

Preparation of 6-Mono[*O,O*-bis(2-aminopropyl)-polypropylene glycol-*block*-poly(ethylene glycol)-*block*-polypropylene glycol]- β -cyclodextrin. 6-Mono(*p*-toluenesulfonyl)- β -cyclodextrin (0.995 g, 0.772 mmol), synthesized according to the procedure reported in the literature (20), was suspended in 35 mL of *O,O*-bis(2-aminopropyl)polypropylene glycol-*block*-poly(ethylene glycol)-*block*-polypropylene glycol (53.34 mmol) (diamino-PEG, Jeffamine ED-600), and a minimum amount of DMF was added to the suspension to obtain a clear solution. The mixture was stirred at 80 °C in a three-necked flask equipped with reflux condenser under argon atmosphere for 8–9 h. The reaction progress was checked by analytical thin-layer chromatography (TLC): 50 μ L of mixture was added to 1 mL of acetone, and the precipitate was collected by centrifugation and washed five times with 1 mL of acetone. The precipitate was dissolved in 100 μ L of methanol and analyzed by TLC plate eluted with ethyl acetate/2-propanol/water/30% ammonia (13:10:5:1.4, volume ratios). Disappearance of

the UV positive spot relative to mono(*p*-toluenesulfonyl)- β -cyclodextrin ($R_f = 0.05$) gave evidence of reaction endpoint, while the spots positive to *p*-anisaldehyde, I₂, and ninhydrin corresponded to the final product 6-mono[*O,O*-bis(2-aminopropyl)polypropylene glycol-*block*-poly(ethylene glycol)-*block*-polypropylene glycol]- β -cyclodextrin ($R_f = 0.006$). The reaction mixture was precipitated in 250 mL of acetone, and the crude product was washed five times with 20 mL of the same solvent, collected by centrifugation, and desiccated under vacuum in the presence of phosphorus pentoxide.

One milligram of CD-PEG-NH₂ in 1 mL of acetonitrile/water/acetic acid (50/50/1, v/v/v) was analyzed by ESI-TOF.

CD-PEG-NH₂ in DMSO-*d*₆ was analyzed by ¹H NMR using a Bruker Spectrospin 300 (300 MHz): δ 5.69–5.58 (br s, OH-2, of β -cyclodextrin, 7H), 5.66 (br s, OH-3 of β -cyclodextrin, 7H), 4.81 (br d, H-1 of β -cyclodextrin, 7H), 4.42 (br s, OH-6 of β -cyclodextrin, 6H), 3.78–3.42 (m, H-6–3–5 of β -cyclodextrin and CH₂CH₂ of PEG, ~72H), 3.40–3.20 (m, H-4 and H-2 of β -cyclodextrin overlapping HOD), 1.08–0.86 (m, propyl CH₃ of Jeffamine ED-600, ~12H).

Synthesis of 6-Mono{folic acid- γ -[*O,O*-bis(2-aminopropyl)polypropylene glycol-*block*-poly(ethylene glycol)-*block*-polypropylene glycol]- β -cyclodextrin. 6-Mono[*O,O*-bis(2-aminopropyl)polypropylene glycol-*block*-poly(ethylene glycol)-*block*-polypropylene glycol]- β -cyclodextrin (0.791 g, 0.438 mol) was added to 50 mL of anhydrous DMSO containing 2.884 g (5.356 mmol) of *N*-hydroxysuccinimide ester of folic acid prepared according to the procedure reported in the literature (5). TEA (320 μ L, 0.2295 mmol) was added to the reaction mixture, and pH 9.0 was maintained by addition of the same base. The reaction was stirred under dark and anhydrous conditions. After 40 h of reaction, the solution was poured dropwise into 500 mL of cold acetone, and the yellow precipitate was washed 10 times with 50 mL of acetone. The crude product was dried under vacuum in the presence of phosphorus pentoxide. The dry product was dissolved in 100 mL of 0.1 M borate buffer, pH 8.3. The solution was fractionated by gel permeation chromatography using Sephadex G25 superfine column eluted with 10 mM ammonium carbonate buffer, pH 8.0. One milliliter fractions were collected and analyzed by UV at 363 nm, iodine test, and *p*-anisaldehyde for folic acid, PEG, and cyclodextrin determination, respectively (18–20). Fractions positive to all tests were pooled and concentrated by ultrafiltration using a 500 Da cut-off membrane. Further purification was carried out using a preparative RP-C18 column (2.5 cm \times 22 cm) operated on a Shimadzu SP-10 HPLC system and eluted in a gradient mode, using 10 mM ammonium acetate pH 6.5 (eluent A) and acetonitrile (eluent B), from 10% to 40% eluent B in 40 min. The UV detector was set at 363 nm. The peak corresponding to the conjugate was collected, concentrated, and desalted by ultrafiltration. The solution was lyophilized and analyzed by RP-C18 chromatography.

UV Analysis. Ten milligrams of β -cyclodextrin-PEG-folic acid, dissolved in 10 mL of 10 mM phosphate buffer, 0.15 M NaCl, pH 7.0, was analyzed by UV. The folate content in the final product was determined on the basis of the folic acid extinction coefficient value reported in the literature (ϵ_M 6.197 M⁻¹ cm⁻¹ at 363 nm and 25820 M⁻¹ cm⁻¹ at 280 nm) (21). The carrier concentrations in stock solutions were determined by UV using the same molar extinction coefficients.

¹H NMR: (300 MHz, DMSO-*d*₆) δ 8.63 (s, C7-H of FA, 1H), 7.64 (d, 2',6'-H of FA, 2H), 6.64 (d, 3',5'-H of FA, 2H), 5.76–5.60 (m, OH-2, OH-3 of β-cyclodextrin, 14H), 4.87–4.76 (br d, H1 of β-cyclodextrin, 7H), 4.52–4.36 (br, OH-6 of β-cyclodextrin, 6H), 4.28–4.16 (m, α-CH₂ of Glu of FA, 1H), 3.78–3.42 (m, H6-3-5 of β-cyclodextrin and CH₂CH₂ of PEG, ~72H), 3.40–3.14 (m, H-4 and H-2 of β-cyclodextrin overlapping HOD), 1.08–0.86 (m, propyl CH₃ Jeffamine ED-600, ~12H).

Solubility Studies. Five milligrams of β-estradiol was added to 0.1 mL of 10 mM phosphate buffer, 0.15 M NaCl, pH 7.2, containing increasing concentrations of β-cyclodextrin (0–16.3 mM) or equimolar amounts of β-cyclodextrin/PEG/folic acid (1:1:1) mixture or CD-PEG-FA (0–42 mM). The suspensions were tumbled gently for 90 h at room temperature and then centrifuged at 10000 rpm for 5 min. The β-cyclodextrin and β-cyclodextrin/PEG/folic acid mixture solutions were diluted 10 times with the same buffer, and β-estradiol was assessed by RP–C18 chromatography isocratically eluted with methanol/water (67:33, v/v), 0.05% trifluoroacetic acid. The UV detector was set to 280 nm. The drug content in the solutions was determined according to the peak area, which was referred to the corresponding calibration plots obtained by analysis of solutions containing different β-estradiol concentrations. The CD-PEG-FA solutions were added to 2 mL of acetone and centrifuged at 10000 rpm for 5 min. The supernatant was desiccated, lyophilized, redissolved in a 67% methanol/water solution, and analyzed by HPLC as reported above.

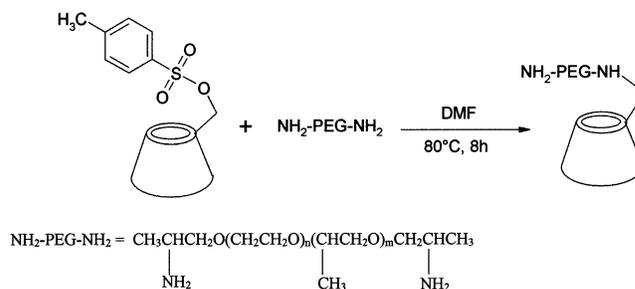
Degradation Studies. Five hundred microliters of 10 mM phosphate buffer, 0.15 M NaCl, pH 7.2, containing 4 mM of β-cyclodextrins or equimolar concentrations of CD-PEG-FA or β-cyclodextrin/PEG/folic acid (1:1:1 molar ratio), was added to 100 μL of chlorambucil solution in methanol (1.2 mM). The solutions were maintained at 22 °C, and chlorambucil was estimated at scheduled times by RP–C18 column isocratically eluted with acetonitrile/water (60:40, v/v), 0.05% TFA, and detected by UV at 302 nm according to the procedure reported in the literature (22).

Hemolysis Assay. One milliliter of heparinized mouse blood was centrifuged at 2000 rpm for 10 min, and the precipitated erythrocytes were washed three times with 10 mM phosphate buffer, 0.15 M NaCl, pH 7.4. The erythrocytes were resuspended in the buffer to give 5% (w/v) hematocrit concentration. Hematocrit samples (30 μL) were added to 570 μL of buffer solutions containing β-cyclodextrin (0–16.3 mM) or equimolar amounts of CD-PEG-FA or β-cyclodextrin/PEG/folic acid 1:1:1 mixture. The samples were incubated at 37 °C for 30 min and then centrifuged at 2000 rpm for 4 min. The released hemoglobin was determined by optical density (OD) at 543 nm and expressed as (sample OD/control OD) × 100. The control corresponded to the samples containing 0 mM product (18).

Molecular Modeling. All simulations were performed by using AMBER 6.0 (23, 24), Cerius² (v.4.2, Accelrys, San Diego, CA), Materials Studio (v. 2.2, Accelrys), Discover (v. 2000, Accelrys), and in-house developed codes (stand-alone and add-on to the commercial software). The all-atom force field (FF) parameters set by Cornell et al. (25) (in *parm94.dat* file of the AMBER 6.0 code) was applied in all calculations.

The procedure for the generation of accurate model structures for β-cyclodextrin and the drugs is reported in details elsewhere (26). Accordingly, only a brief account

Scheme 1



will be given here. The accurate model structures for the CD-PEG-FA and the drug compounds were generated and subjected to an initial energy minimization using Discover, applying a convergence criterion of 10⁻⁴ kcal/(mol Å). The conformational search was carried out using a combined molecular mechanics/molecular dynamics simulated annealing (MDSA) protocol (27, 28). Accordingly, the relaxed structures were subjected to five repeated temperature cycles (from 310 to 1000 K and back) using constant volume/constant temperature (NVT) MD conditions. At the end of each annealing cycle, the structures were again energy minimized to converge below 10⁻⁴ kcal/(mol Å), and only the structures corresponding to the minimum energy were used for further modeling. The electrostatic charges for the geometrically optimized drug molecules were obtained by restrained electrostatic potential fitting (29), and the electrostatic potentials were produced by single-point quantum mechanical calculations at the Hartree–Fock level with a 6-31G* basis set. All ab initio calculations were carried out with DMol³ (30), as implemented in the Cerius² modeling suite.

For the MD simulations, each given system was solvated by a cubic box of TIP3P water molecules (31), extended at least 10 Å in each direction from the solute. All water molecules of which the oxygen atom lied within 2.3 Å of a non-hydrogen β-cyclodextrin atom were removed. The periodic boundary conditions with constant pressure of 1 atm were applied, and long-range non-bonded van der Waals interactions were truncated by using a 8 Å residue-based cutoff. The particle mesh Ewald (PME) method (32) was used to treat the long-range electrostatics. Each system was gradually heated to 310 K in three intervals, allowing a 5 ps interval per each 100 K and then equilibrated for 25 ps at 310 K, followed by 2.0 ns of data collection runs. An integration time step of 2 fs has been used with constant temperature, being the temperature maintained at a constant value by the Berendsen coupling algorithm (33), with separate solute–solvent and solvent–solvent coupling.

RESULTS

Preparation and Characterization of Folic Acid Conjugates. The preparation of the monosubstituted cyclodextrin–PEG–folic acid conjugate (CD-PEG-FA) was carried out by a two-step procedure: (1) synthesis of CD-PEG-NH₂; (2) synthesis of CD-PEG-FA. To obtain a derivative constituted by β-cyclodextrin, poly(ethylene glycol), and folic acid in the molar ratio of 1:1:1, monotosylated-β-cyclodextrin was used as starting product. The reaction of monotosylated-β-cyclodextrin, Scheme 1, with a 60-fold excess of 700 Da diamino-PEG allowed for β-cyclodextrin monosubstitution (CD-PEG-NH₂) without formation of heterogeneous cross-linking products which could be formed because of the bifunctional nature of the diamino-PEG. The chemical structure of Jeffamine ED-

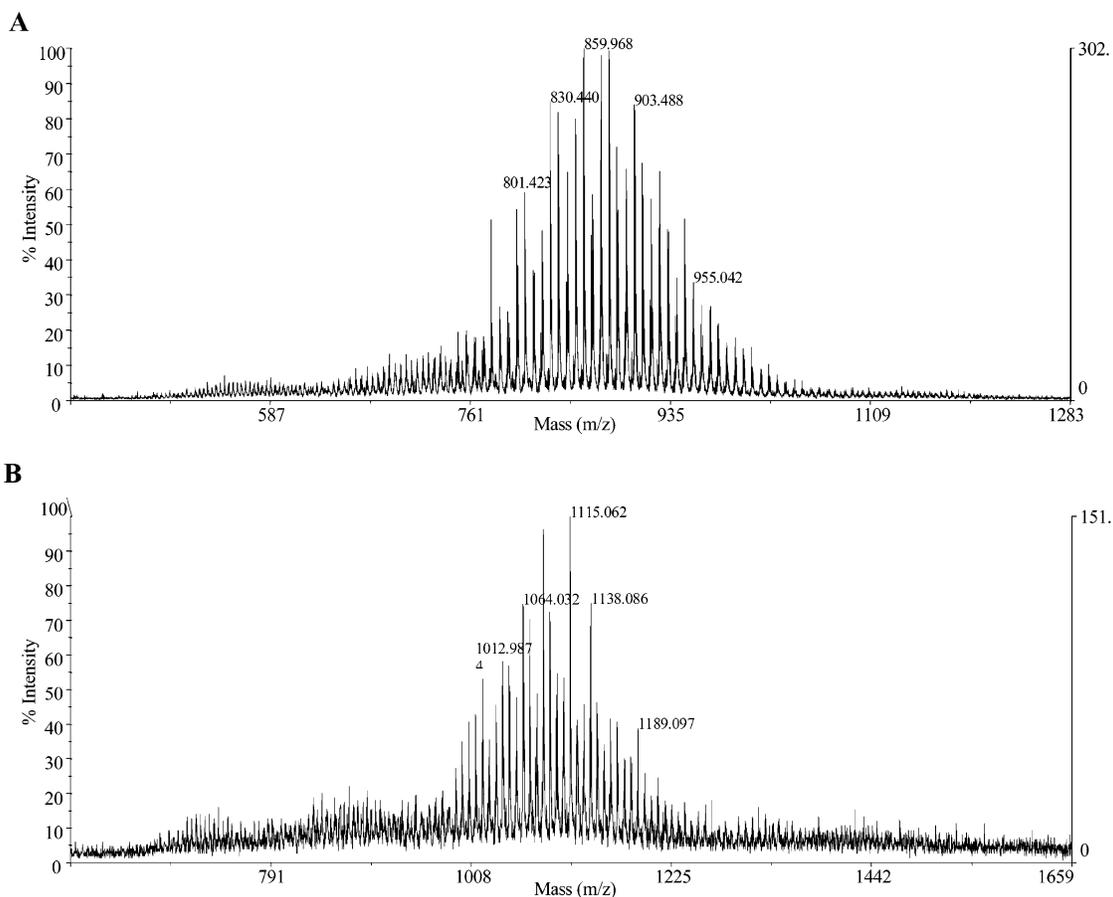


Figure 1. ESI-TOF mass spectra: (A) β -cyclodextrin-PEG; (B) β -cyclodextrin-PEG-folic acid.

600 (diamino-PEG) reported in Scheme 1 shows that this oligomer is composed of $(\text{OCH}_2\text{CH}_2)$ units with 44 Da molecular weight and two terminal polypropylene glycol-aminopropyl groups. A preliminary mass spectrometry analysis showed that this oligomer has a molecular weight of about 700 Da.

The TLC analysis showed that under the selected coupling conditions the reaction was complete in 8 h as neither UV- nor *p*-anisaldehyde-positive spots relative to unreacted 6-mono(*p*-toluenesulfonyl- β -cyclodextrin) and β -cyclodextrin, respectively, were detected. The ninhydrin-, iodine-, and *p*-anisaldehyde-positive spot corresponded to the final CD-PEG-NH₂ product. After acetone precipitation, the product recovery yield was of 72% (obtained weight/expected weight, %).

Figure 1A shows that the mass spectrum obtained with the CD-PEG-NH₂ conjugate exhibits a bell shaped distribution characterized by 22 *m/z*-spaced lines centered at 900 *m/z*. The 900 *m/z* signal has been assigned to $[\text{CD-PEG-NH}_2 + 2\text{H}^+]^{2+}$ (CD-PEG-NH₂ calculated molecular mass 1805 Da). The 22 *m/z* (44 Da) mass difference between the signals corresponds to the oxyethylene unit ($\text{CH}_2\text{CH}_2\text{O}$), indicating that the cluster was due to the PEG heterogeneity.

The ¹H NMR analysis of the derivative showed the signals typical of β -cyclodextrins [δ 5.69–5.58 (br s, OH-2, of β -cyclodextrins, 7H), 5.66 (br s, OH-3 of β -cyclodextrins, 7H), 4.81 (br d, H-1 of β -cyclodextrins, 7H)] and polypropylene glycol-*block*-poly(ethylene glycol)-*block*-polypropylene glycol [δ 3.78–3.42 (m, H-6-3-5 of β -cyclodextrins and CH_2CH_2 of PEG, ~72H), 1.08–0.86 (m, propyl CH_3 of Jeffamine ED-600, ~12H)] which corresponded to the values reported in the literature (34). The monosubstitution of β -cyclodextrin with PEG was

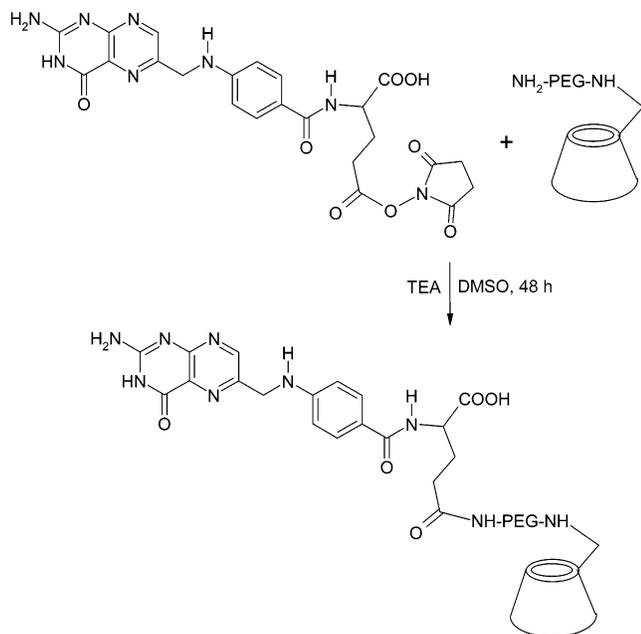
demonstrated by the H integration values of the β -cyclodextrin primary hydroxyl group [δ 4.48–4.32 (d, OH-6 of β -cyclodextrin, 6H)] and by the integration value ratios between the signal corresponding to the β -cyclodextrin and Jeffamine ED-600.

CD-PEG-FA was prepared by reaction of CD-PEG-NH₂ with excess of folic acid, which was activated as the succinimidyl ester in the presence of dicyclohexylcarbodiimide according to the procedure reported in the literature (5). The activation procedure yields 80% γ -carboxyl group and 20% α -carboxyl group of the glutamate residue activation. The CD-PEG-NH₂ reaction with excess of activated folic acid (12:1 activated folic acid/CD-PEG-NH₂ molar ratio) yielded extensive CD-PEG-NH₂ derivatization in 40 h as determined by ninhydrin assay. The reaction procedure to obtain CD-PEG-FA is represented in Scheme 2.

The extensive CD-PEG-NH₂ derivatization avoided purification problems due to the separation of unreacted CD-PEG-NH₂ from CD-PEG-FA, which present similar physicochemical properties. The final product was purified from the residual folic acid and hydroxysuccinimide by gel permeation. Small amounts of free folic acid were found in the fractions containing CD-PEG-FA (positive to iodine test, *p*-anisaldehyde test and UV) probably because of the formation of folic acid/ β -cyclodextrin inclusion complex or macromolecular aggregates. Complete elimination of free folic acid was achieved by reversed-phase chromatography, though CD-PEG-FA was eluted with a broad peak due to the PEG polydispersity. At the end of the two-step purification, the product yield was 38.6% (w/w).

Figure 1B shows the mass pattern of the conjugate, which exhibits a bell-shaped distribution of 22 *m/z*-spaced

Scheme 2



signals centered at 1100 m/z (calculated molecular mass 2229 Da). The signal m/z has been assigned to $[\text{CD-PEG-FA} + 2\text{H}^+]^{2+}$.

The ^1H NMR showed the signals corresponding to CD [δ 5.76–5.60 (m, OH-2, OH-3 of β -cyclodextrins, 14H), 4.87–4.76 (br d, H-1 of β -cyclodextrins, 7H), 4.52–4.36 (d, OH-6 of cyclodextrins, 6H)], PEG [δ 3.78–3.42 (m, H-6-3-5 of β -cyclodextrins and CH_2CH_2 PEG, \sim 72H), 1.08–0.86 (m, propyl CH_3 of Jeffamine ED-600, \sim 12H)], and folic acid [δ 8.63 (s, C7-H of FA, 1H), 7.64 (d, 2',6' H of FA, 2H), 6.64 (d, 3',5' H of FA, 2H), 4.28–4.16 (m, α - CH_2 of Glu of FA, 1H)]. The results confirmed the chemical CD-PEG-FA conjugate identity and composition (1:1:1 β -cyclodextrin/poly(ethylene glycol)/folic acid molar ratio).

Solubility Studies. The ability of the CD-PEG-FA conjugate to form inclusion complexes with drugs was investigated according to the method reported by Higuchi and Connors (35). β -Estradiol was selected as model drug since its solubility was demonstrated to increase significantly by interaction with β -cyclodextrin. For comparison, β -estradiol phase solubility diagrams were obtained also in the presence of unmodified β -cyclodextrins, and β -cyclodextrin/diaminoPEG/folic acid 1:1:1 mixture.

The results depicted in Figure 2 show that the β -estradiol solubility in the presence of CD-PEG-FA conjugate is 540 and 8 times higher as compared to the solubility in physiologic buffer (3 $\mu\text{g}/\text{mL}$) and in β -cyclodextrins solution, respectively.

The apparent stability constant (K_c) for β -estradiol complex was calculated by the slope of the phase-solubility diagram according to the equation:

$$K_c = \frac{\text{slope}}{S_0(1 - \text{slope})}$$

where S_0 is the saturation concentration of β -estradiol in buffer without cyclodextrin (3 $\mu\text{g}/\text{mL}$) (36). The stability constants of β -estradiol for CD-PEG-FA, β -cyclodextrins, and β -cyclodextrin/PEG/folic acid mixture reported in Table 1 indicate that the presence of PEG/folic acid decreases the drug affinity for the β -cyclodextrins about

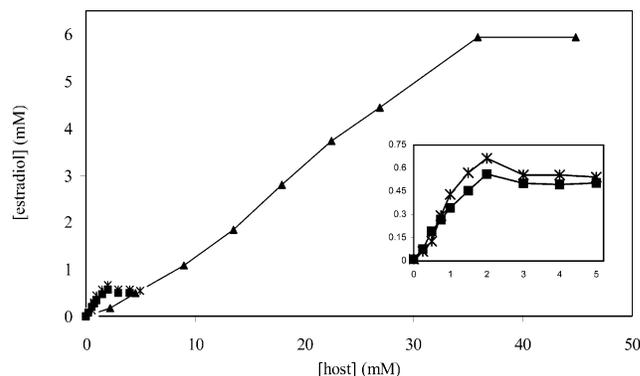


Figure 2. Phase-solubility diagram of estradiol in PBS, pH 7.2 at increasing concentration of β -cyclodextrin (*), β -cyclodextrin/PEG/folic acid 1:1:1 molar ratio mixture (■), and CD-PEG-FA (▲). The inset contains a magnified phase-solubility diagram of β -estradiol in the presence of β -cyclodextrins and β -cyclodextrin/PEG/folic acid mixture.

Table 1. β -Estradiol Inclusion Parameters and Chlorambucil Degradation Kinetic Constants in β -Cyclodextrin, β -Cyclodextrin/PEG/Folic Acid Mixture, and CD-PEG-FA

β -Estradiol Inclusion Constants	
	K_c (M^{-1})
β -cyclodextrin	77947
β -cyclodextrin/PEG/folic acid 1:1:1 mol ratio mixture	54221
CD-PEG-FA	15343
Chlorambucil Degradation Kinetic Constants	
	$K_{\text{obsd}} \times 10^{-4}$ (min^{-1})
chlorambucil	11.01
chlorambucil/ β -cyclodextrin mixture	0.85
chlorambucil/ β -cyclodextrin/PEG/folic acid mixture	1.88
chlorambucil/CD-PEG-FA mixture	8.38

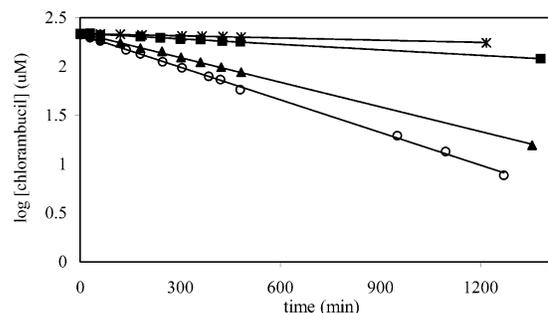


Figure 3. Degradation profile of chlorambucil (200 μM) in PBS, pH 7.2: chlorambucil (○); chlorambucil in the presence of β -cyclodextrin (*); chlorambucil in the presence of β -cyclodextrin/PEG/folic acid 1:1:1 molar ratio mixture (■); chlorambucil in the presence of CD-PEG-FA (▲).

30% while the conjugation of PEG and folic acid reduces the β -estradiol affinity about 75%.

Stability Properties. The degradation profiles of chlorambucil in the presence of CD-PEG-FA, unmodified β -cyclodextrins, and β -cyclodextrin/PEG/folic acid mixture 1:1:1 reported in Figure 3 show that in any case chlorambucil undergoes first-order degradation kinetics. In physiologic buffer, the free drug undergoes rapid degradation while in the presence of unmodified β -cyclodextrins, the drug displays high stability. In the last cases, 20% of the drug amount was degraded in 24 h. CD-PEG-FA was found to partially prevent drug degradation, although the protective effect was significantly lower than that dis-

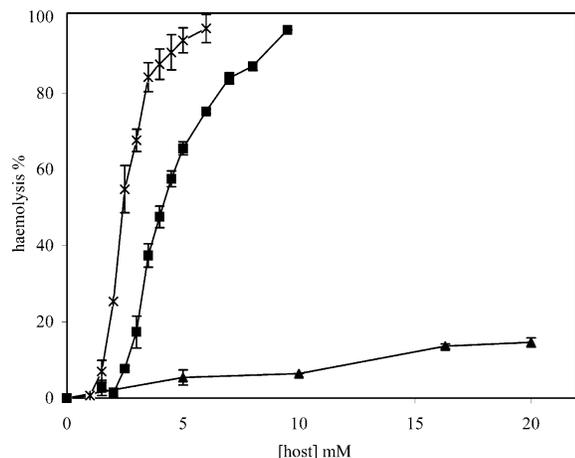


Figure 4. Hemolytic effects of β -cyclodextrins on mouse erythrocytes in phosphate-buffered saline (pH 7.4) at 37 °C: β -cyclodextrin (*); β -cyclodextrin/PEG/folic acid 1:1:1 molar ratio mixture (■); CD-PEG-FA (▲). Each point represents the mean of four experiments.

played by native β -cyclodextrins. The degradation rate constants of chlorambucil reported in Table 1 indicate that the protective effect of the CD-PEG-FA derivative is about 10 times lower than in the case of β -cyclodextrins.

Hemolysis Activity. The hemolytic properties of CD-PEG-FA were investigated by spectrophotometric evaluation of the hemoglobin released from erythrocytes incubated with increasing amounts of conjugate. For comparison, the test was carried out using equimolar amounts of β -cyclodextrins and β -cyclodextrin/PEG/folic acid 1:1:1 molar ratio mixtures.

According to the literature data, Figure 4 shows that β -cyclodextrins possess strong hemolytic activity, which decreases of about 50% by folic acid addition to the β -cyclodextrin/PEG mixture. The conjugate displayed negligible hemolytic character as compared to the native β -cyclodextrins or to the β -cyclodextrin/PEG/folic acid mixture also when high bioconjugated β -cyclodextrin concentrations were used.

DISCUSSION

The synthetic procedure adopted in the present research allowed for obtaining a new β -cyclodextrin–poly(ethylene glycol)–folic acid bioconjugate, which can behave as a Trojan horse for active drug delivery. The hydrophobic pocket of cyclodextrins, which allows for inclusion of many lipophilic molecules, can allocate molecules to be released into specific tissues. The availability of a wide array of natural and semisynthetic cyclodextrins with different physicochemical properties represents a further advantage in the use of these polysaccharides for preparation of tailor-made drug delivery systems. Nevertheless, cyclodextrins do not have per se targeting properties; therefore, the conjugation of a targeting moiety is an essential requisite to develop systems for active delivery. For such a reason, folic acid conjugation was addressed to endow a β -cyclodextrin derivative which can be actively recognized by folic acid receptor overexpressing tumor cells. PEG was used as spacer arm because it confers flexibility to folic acid, allowing for its receptor interaction, and avoids problems related to the cyclodextrin steric hindrance. Actually, many studies demonstrated that PEG with a molecular weight higher than 2000 Da is required to achieve targetable colloidal systems (nanoparticles, liposomes)

(37). However, in the case of β -cyclodextrin we selected a shorter spacer in consideration of the lower hindrance of this small carrier.

The solubility studies carried out with β -estradiol demonstrated that the conjugate maintains the β -cyclodextrin ability to form inclusion complexes with hydrophobic drugs, although the solubilization properties of the β -cyclodextrin were strongly altered by PEG–folic acid conjugation. In particular, the higher solubility of β -estradiol in the presence of the conjugate with respect to the β -cyclodextrins could be attributable to the high solubility of carrier (CD-PEG-FA), which increases the complex solubility. CD-PEG-FA was in fact about three times more soluble than the unmodified β -cyclodextrins probably because of the presence of the freely soluble PEG and the epta-glucopyranose ring modification, which avoids the β -cyclodextrin precipitation. However, the affinity of β -estradiol for the conjugated β -cyclodextrins was lower than for the unmodified cyclo-polysaccharides.

The phase solubility diagram of β -estradiol with β -cyclodextrin or β -cyclodextrin/PEG/folic acid mixture resulted in a B-type Higuchi phase solubility diagram, both showing a good linear regression (R^2 of 0.96 and 0.98 for β -cyclodextrin and β -cyclodextrin/PEG/folic acid mixture, respectively). In both cases, further addition of cyclodextrins over 2 mM did not modify the final β -estradiol solubility since the β -cyclodextrin/ β -estradiol complex solubility end point is reached. On the other hand, the phase solubility diagram of β -estradiol with β -cyclodextrin-PEG-folic acid resulted in an A_p -type Higuchi phase solubility diagram (35, 38). Therefore, the β -cyclodextrin/ β -estradiol complex is characterized by a $[\text{carrier}]_1: [\text{drug}]_1$ complexation order while the CD-PEG-FA/ β -estradiol complex is characterized by $[\text{carrier}]_x: [\text{drug}]_1$ complexation order. These findings are supported by a molecular dynamics study performed on the CD-PEG-FA/ β -estradiol complex, for which a 2:1 stoichiometry was assumed, reveals that the drug is not fully included in the cyclodextrin cavities, but gives rise to a sandwichlike molecular assembly, leading to drug dissolution (see Figure 5).

Although the phase solubility results and molecular dynamics study indicate that the β -cyclodextrin–PEG–folic acid/ β -estradiol complexation takes place with $[\text{carrier}]_x: [\text{drug}]_1$ stoichiometric order, the complex model was simplified to a 1:1 stoichiometric ratio to calculate the complex constant stability (35). Similar elaborations were also reported in the literature for solubility phase diagrams obtained with cholesterol and hydroxypropyl- β -cyclodextrin (38).

The decrease in affinity constant of β -estradiol may be sensibly ascribed to a combination of events, such as folic acid competition for the β -cyclodextrins cavity and structural modification of the cyclo-glucopyranose unit. Interestingly it was demonstrated that the reduction in affinity was not due to the PEG inclusion into the cyclo-polysaccharide cavity, although the literature reports that pluronic can be included into β -cyclodextrins forming macromolecular structure named polyrotaxanes (39). Nevertheless, Jeffamine has only a short chain portion (end-chain propyl group) similar to the pluronic structure. On the contrary, the in vitro studies demonstrated that the addition of free folic acid significantly decreases the drug complexation, indicating that β -estradiol competes with folic acid in forming inclusion complexes.

This observation is supported by the MD simulations performed on the CD-PEG-FA molecule in water, of which Figure 6 reports three snapshots. The analysis of the MD trajectories reveals that the PEG chain, by virtue

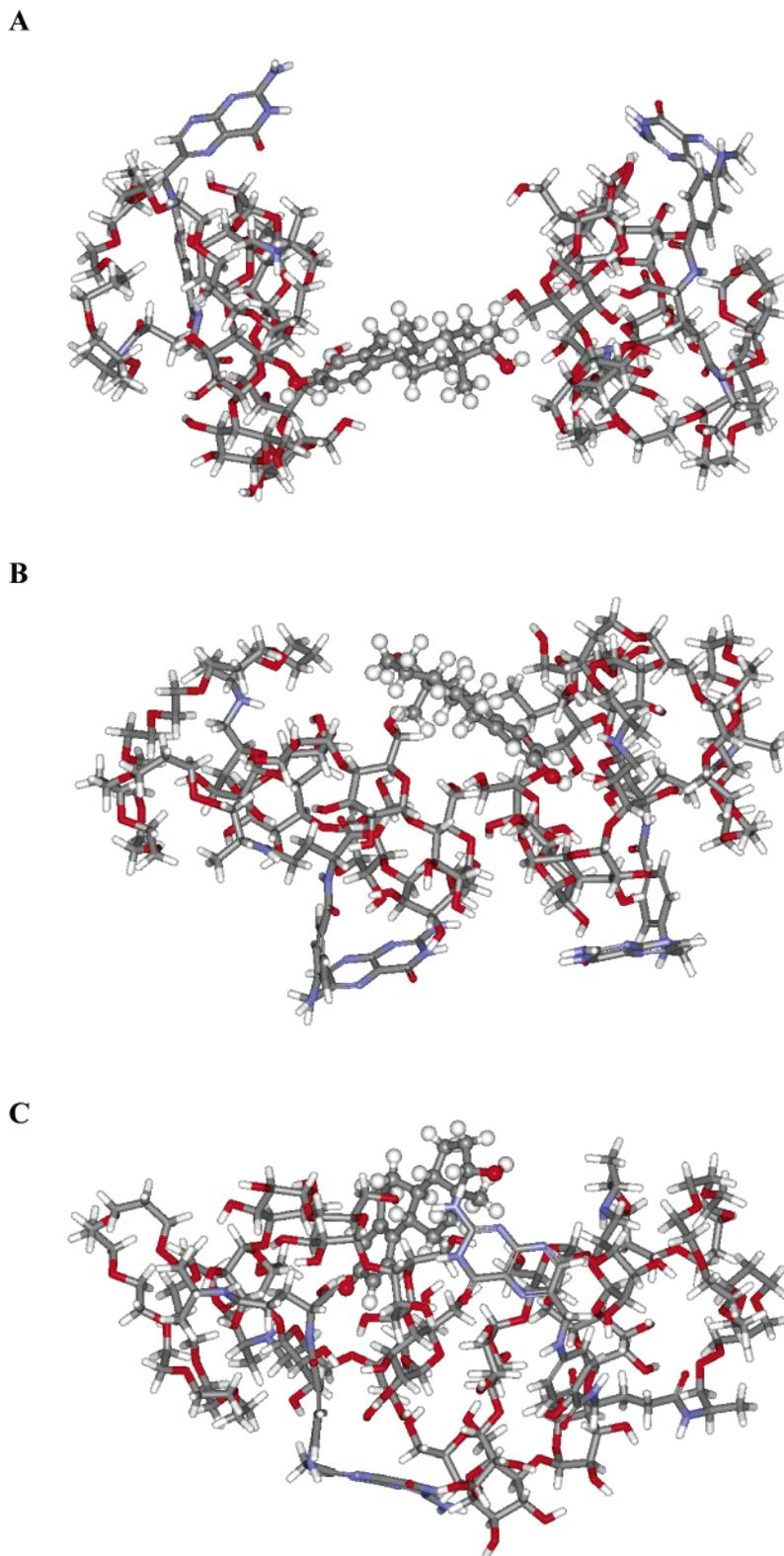


Figure 5. Three snapshots of the MD simulation performed for 2 ns at 310 K on the 1:2 β -estradiol/CD-PEG-FA assembly in water. (A) $t = 0$; (B): $t = 1$ ns; (C): $t = 2$ ns. For clarity, β -estradiol is shown in ball-and-stick representation, CD-PEG-FA is shown in stick representation, and water molecules have been omitted.

of its intrinsic flexibility, partially folds up and allows the carboxylic group of the folic acid moiety to enter the cyclodextrin cavity, thereby remaining for the entire course of the simulation. This notable affinity for the self-inclusion of the folic acid undoubtedly constitutes a hindrance for the “canonical” inclusion mechanism of drugs into this cyclodextrin bioconjugate and can be taken as one of the partial factors responsible for the

lower affinity of β -estradiol toward CD-PEG-FA with respect to the parent cyclodextrin. Moreover, the simulation conditions employed here mimic an infinite dilution condition; accordingly, phenomena such as cyclodextrin–cyclodextrin intermolecular interactions taking place, resulting in the inclusion of one folic acid moiety into the cyclodextrin cavity of another carrier molecule, have not been considered. This could also account for the relevant

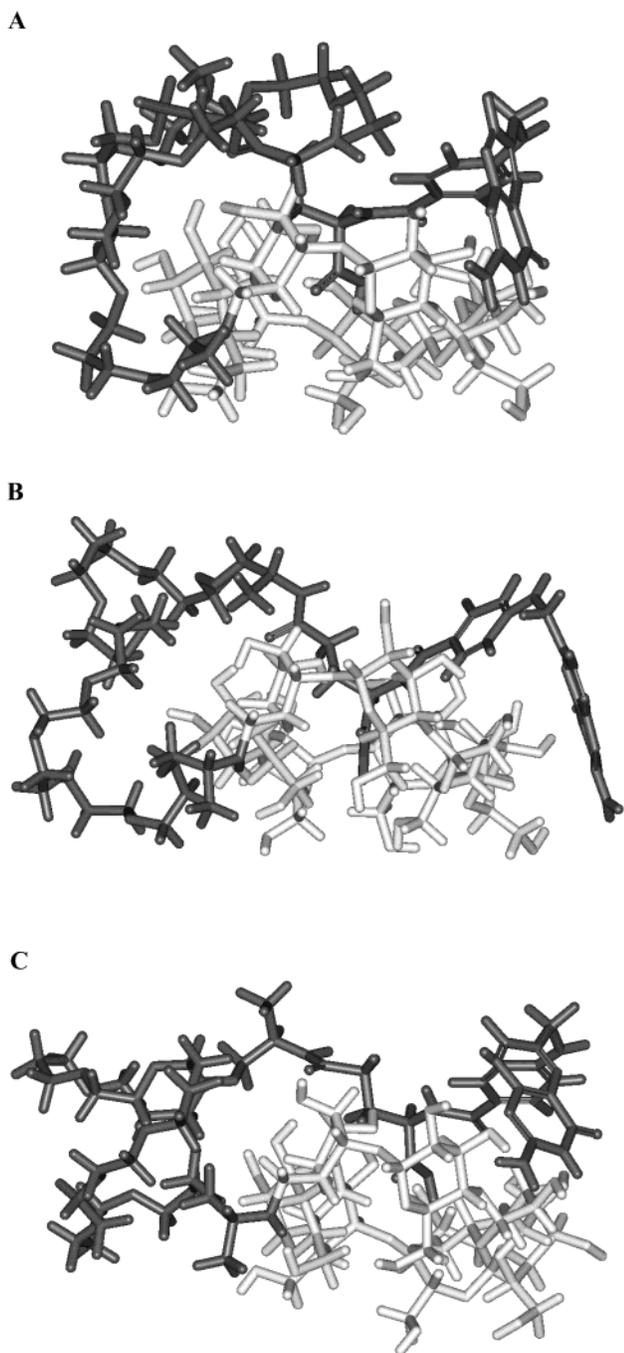


Figure 6. Three snapshots taken from the MD trajectory of CD-PEG-FA in water. (A): $t = 0.5$ ns; (B): $t = 1$ ns; (C): $t = 2$ ns. For clarity, the carbohydrate macrocycle is colored light gray, the PEG-FA substituents chain is colored dark gray, and water molecules have been omitted.

decrease of β -estradiol affinity constant, as high conjugated cyclodextrin concentrations were used in the corresponding experiments. On the other hand, this last event should be limited in the body, where very low concentration of conjugate is obtained and intermolecular displacement phenomena should be quite limited.

The lower affinity constant of β -estradiol for the conjugate with respect to the β -cyclodextrin/PEG/folic acid mixture indicates that the partial inclusion of folic acid is not the only reason of the decrease in drug complexation. Also the considerable modification of the epta-glucopyranose ring induced by the presence of the mobile PEG chain, as can be envisaged from Figure 6, plays a relevant role in preventing the inclusion of this

drug, characterized by a flat but sterically large molecular shape.

Chlorambucil is a labile drug, which undergoes first-order kinetic hydrolysis (22). Degradation kinetic order was neither affected by the presence of β -cyclodextrin or CD-PEG-FA. However, PEG-folic acid conjugation was found to decrease significantly the ability of β -cyclodextrins to prevent the chlorambucil degradation. The similar chlorambucil degradation profiles obtained by drug incubation in buffer or in the presence of the β -cyclodextrin conjugate indicate that the modified carbohydrate macrocycle is not effective in preventing the degradation of this labile drug.

From the standpoint of molecular modeling, the possibility for chlorambucil to form stable inclusion complexes with CD-PEG-FA is suggested by several factors, such as limited drug molecular dimensions and presence of a central, disubstituted phenyl ring. Further, experimental evidence of 1:1 inclusion complexes between chlorambucil and native β -cyclodextrin have appeared in the literature (17). In this case, the aim of our MD simulations was to follow the dynamics of the inclusion complex formation and the behavior of the resulting supramolecular assembly during a 2 ns time period. Figure 7 shows the minimized, starting structure of the molecular assembly plus five snapshots taken from the corresponding molecular system trajectory. During the equilibration phase, the drug moves toward the cavity of CD-PEG-FA. Figure 7B shows the drug closer to the cavity with respect to the energy minimized, initial situation (Figure 7A). During the first half of dynamic simulation, the drug tends to position parallel to the cavity of the macrocycle, with the acetyl group within the cavity (0.5–1 ns, Figures 7C and 7D). Around 1.5 ns of simulation, the drug is aligned with the cavity axis and with the phenyl ring well inserted in the host (Figure 7E). The last part of the simulated period shows the slow drug release from the cavity, mainly due to the partial insertion of the folic acid into the cavity itself (Figure 7F). Since the same simulation performed on a chlorambucil/unmodified β -cyclodextrin system (not shown) leads to a stable complex, in which the guest is well inserted within the carbohydrate host, the observed behavior in the case of the bioconjugate carrier may partly account for a higher degree of drug degradation.

Typically, β -cyclodextrins display a dramatic hemolytic effects due to membrane cholesterol extraction and inclusion into the β -cyclodextrin cavity (14). The reduction in hemolytic effect observed with β -cyclodextrin/PEG/folic acid and CD-PEG-FA can be explained on the basis of the decreased affinity of β -cyclodextrin for cholesterol. Similarly to what was reported for β -estradiol, the folic acid competition with cholesterol in forming inclusion complexes can be reflected in reduced cell membrane destabilizations and hemoglobin release. The modification of the cyclo-epta-glucopyranose ring further decreases the affinity of cholesterol for the β -cyclodextrin cavity, dramatically reducing the hemolytic properties of this molecule. Therefore, PEG-folic acid conjugation has a significant effect in preventing nonspecific cell damage.

CONCLUSIONS

Recent advances in modern medicinal chemistry and pharmacology point out the need for development of new carriers for active drug delivery, which enhance their therapeutic value. Actually, a number of new drugs are characterized by high pharmacological activity but relevant systemic toxicity, which limits their therapeutic application.

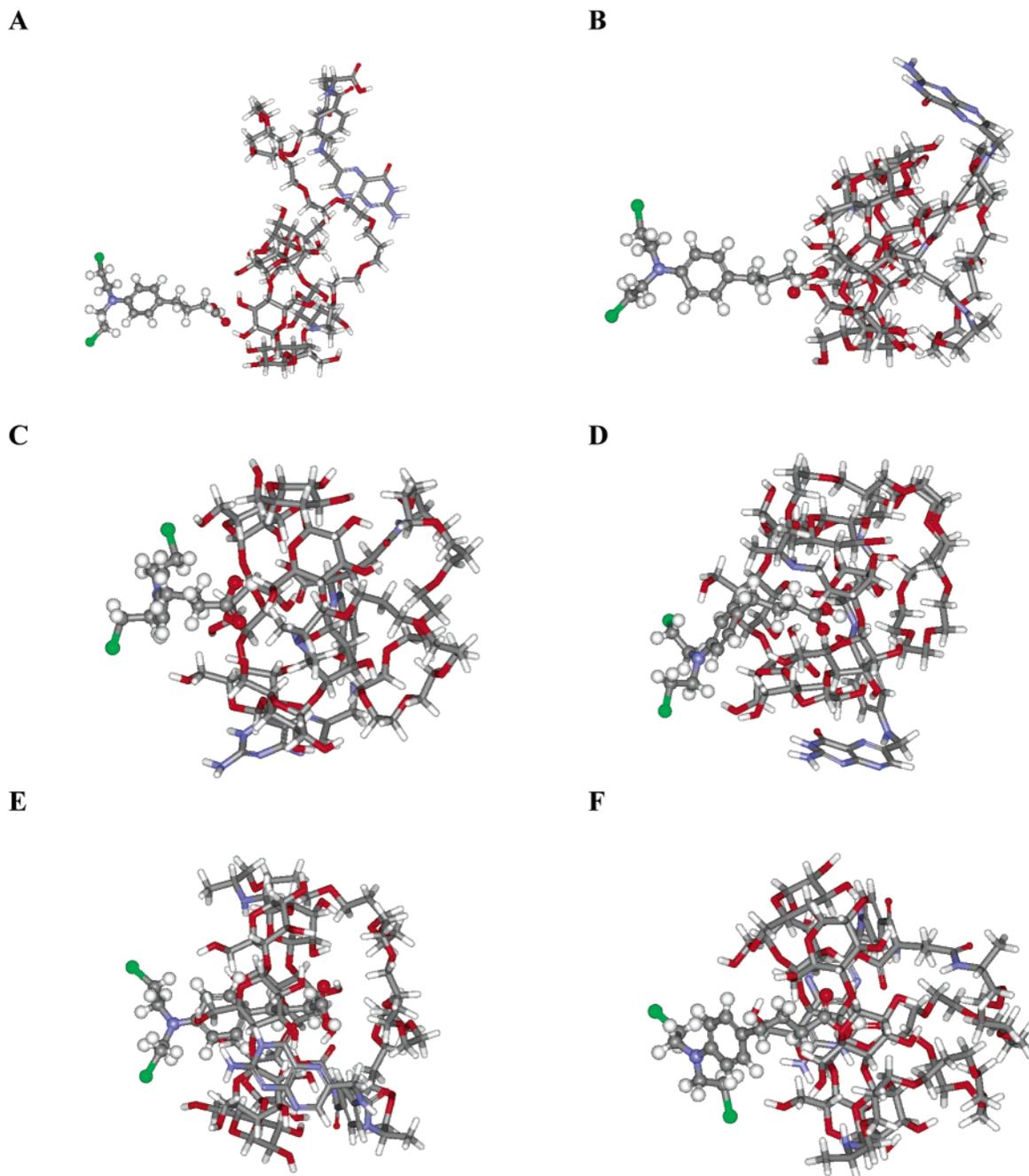


Figure 7. Energy minimized structure and five snapshots extracted from the molecular dynamics trajectory of the chlorambucil/CD-PEG-FA assembly in water at 310K. (A): energy minimized structure; (B): $t = 0$; (C): $t = 0.5$ ns; (D): $t = 1$ ns; (E) $t = 1.5$ ns; (F): $t = 2$ ns. For clarity, chlorambucil is shown in ball-and-stick representation, CD-PEG-FA is shown in stick representation, and water molecules have been omitted.

A suitable strategy to prepare tailor-made targetable drug carriers is the assembly of molecules with peculiar physicochemical and biological properties. In the present study a useful synthetic strategy to obtain a new class of targetable drug carriers has been reported. Cyclodextrins, PEG, and folic acid were assembled to produce a derivative characterized by low molecular weight, high solubility, drug complexation properties, and specific recognition. The results demonstrated that the bioconjugate can form inclusion complexes with hydrophobic drugs allowing for their active delivery to the disease sites.

Finally, it is important to note that the synthetic procedure proposed in the present study can be exploited to prepare a wide array of conjugates. Indeed, bioconju-

gates with different physicochemical, biological, and biopharmaceutical properties can be obtained by proper selection of the cyclo-polysaccharide unit, PEG molecular weight, and targeting moiety.

ACKNOWLEDGMENT

This research was partially supported by MIUR (fondi ex-40%) and CNR.

LITERATURE CITED

- (1) Sudimack, J., and Lee, R. J. (2000) Targeted drug delivery via the folate receptor. *Adv. Drug Del. Rev.* 41, 147–162.
- (2) Turek, J. J., Leamon, C. P., and Low, P. S. (1993) Endocytosis of folate-protein conjugates: ultrastructural localization in KB cells. *J. Cell. Sci.* 106, 423–430.

- (3) Garin-Chesa, P., Campbell, I., Saigo, P. E., Lewis, J. L., Jr., Old, L. J., and Rettig, W. J. (1993) Trophoblast and ovarian cancer antigen LK26. Sensitivity and specificity in immunopathology and molecular identification as a folate-binding protein. *Am. J. Pathol.* *142*, 557–567.
- (4) Wang, S., Lee, R. J., Mathias, C. J., Green, M. A., and Low, P. S. (1996) Synthesis, purification, and tumor cell uptake of ⁶⁷Ga-deferoxamine-folate, a potential radiopharmaceutical for tumor imaging. *Bioconjugate Chem.* *7*, 56–62.
- (5) Lee, R. J., and Low, P. S. (1994) Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis. *J. Biol. Chem.* *269*, 3198–3204.
- (6) Gottschalk, S., Cristiano, R. J., Smith, L. C., and Woo, S. L. (1994) Folate receptor mediated DNA delivery into tumor cells: potosomal disruption results in enhanced gene expression. *Gene Ther.* *1*, 185–191.
- (7) Leamon, C. P., and Low, P. S. (1994) Selective targeting of malignant cells with cytotoxin-folate conjugates. *J. Drug Target.* *2*, 101–112.
- (8) Kataoka, K. (1997) Targetable polymeric drugs. *Controlled drug delivery challenges and strategies*. (K. Park, Ed.) p 49, American Chemical Society, Washington, D.C.
- (9) Jain, R. K. (1990) Physiological barriers to delivery monoclonal antibodies and other macromolecules to tumors. *Cancer Res.* *50*, 814s–819s.
- (10) Castillo, J. A., Palomo-Canales, J., Garcia, J. J., Lastres, J. L., Bolas, F., and Torrado, J. J. (1999) Preparation and characterization of albendazole beta-cyclodextrin complexes. *Drug Dev. Ind. Pharm.* *25*, 1241–1248.
- (11) Hirayama, F., and Uekama, K. (1999) Cyclodextrin-based controlled drug release system, *Adv Drug Del. Rev.* *36*, 125–141.
- (12) Szejtli, J. (1994) Medicinal applications of cyclodextrins. *Med. Res. Rev.* *14*, 353–386.
- (13) Cho, M. J., Chen, F. J., and Huczek, D. L. (1995) Effects of inclusion complexation on the transepithelial transport of a lipophilic substance in vitro. *Pharm. Res.* *12*, 560–564.
- (14) Szentei, L., and Szejtli, J. (1999) Highly soluble cyclodextrin derivatives: chemistry, properties, and trends in development. *Adv. Drug Del. Rev.* *36*, 17–28.
- (15) Bekers, O., Beijnen, J. H., Groot Bramel, E. H., Otagiri, M., Bult, A., and Underberg, W. J. M. (1989) Stabilization of mitomycins on complexation with cyclodextrins in aqueous acidic media. *Int. J. Pharm.* *52*, 239–248.
- (16) Måsson, M., Pitha, J., and Loftsson, T. (1999) Synthesis of cyclic glycerol ether cyclodextrin derivatives and investigation of their binding properties with drugs. *J. Incl. Phenom. Macrocycl. Chem.* *33*, 459–467.
- (17) Måsson, M., Loftsson, T., Jónsdóttir, S., Fridriksdóttir, H., and Petersen, D. S. (1998) Stabilisation of ionic drugs through complexation with nonionic and ionic cyclodextrins. *Int. J. Pharm.* *164*, 45–55.
- (18) Ohtani, Y., Irie, T., Uekama, K., Fukunaga, K., and Pitha, J. (1989) Differential effects of alpha-, beta- and gamma-cyclodextrins on human erythrocytes. *Eur. J. Biochem.* *186*, 17–22.
- (19) Topchieva, I. N., Elezkaya, S. V., Polyakov, V. A., and Karezin, K. I. (1996) Cyclodextrins modified with poly(ethylene oxide) as complexation and transport agents. *Abstracts of the 8th International Symposium on Cyclodextrins*, Budapest, Hungary, pp 2–20.
- (20) Petter, R. C., Salek, J. S., Sikorski, C. T., Kumaravel, G., and Fu-Tyan, L. (1990) Cooperative binding by aggregated Mono-6-(alkylamino)- β -cyclodextrins. *J. Am. Chem. Soc.* *112*, 3860–3868.
- (21) Kranz, D. M., Patrick, T. A., Brigle, K. E., Spinella, M. J., and Roy, J. (1995) Conjugates of folate and anti-T-receptor antibodies specifically target folate-receptor-positive tumor cells for lysis. *Proc. Natl. Acad. Sci.* *92*, 9057–9061.
- (22) Klaus, F., Ed. (1987) *Analytical profiles of Drug Substances*, Vol. 16, pp 85–118, Academic Press Inc., New York.
- (23) Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. S., Cheatham, T. E., III, DeBolt, S., Ferguson, D., Seibel, G. L., and Kollman, P. A. (1995) AMBER, a package of computer programs for applying molecular mechanics, normal-mode analysis, molecular dynamics and free energy calculations to simulate the structural and energetic properties of molecules. *Comput. Phys. Commun.* *91*, 1–41.
- (24) Case, D. A., Pearlman, D. A., Caldwell, J. W., Cheatham, T. E., III, Ross, W. S., Simmerling, C. L., Darden, T. A., Merz, K. M., Stanton, R. V., Cheng, A. L., Vincent, J. J., Crowley, M., Tsui, V., Radmer, R. J., Duan, Y., Pitner, J., Massova, I., Seibel, G. L., Singh, U. C., Weiner, P. K., and Kollman, P. A. (1999). AMBER 6, University of California, San Francisco, CA.
- (25) Cornell, W. D., Cieplak, P., Bayly, C. I., Gould, I. R., Merz, K. M., Jr., Ferguson, D. M., Spellmeyer, D. C., Fox, T., Caldwell, J. W., and Kollman, P. A. (1995) A second generation force field for the simulation of proteins, nucleic acids and organic molecules. *J. Am. Chem. Soc.* *117*, 5179–5197.
- (26) Fermeglia, M., Ferrone, M., Lodi, A., and Pricl, S. (2003) Host-guest inclusion complexes between anticancer drugs and β -cyclodextrin: computational studies. *Carbohydr Polym.* *53*, 15–44.
- (27) Fermeglia, M., Ferrone, M., and Pricl, S. (2002) Computer-aided simulation of a dendrimer with a protoporphyrin core as potential, novel hemoprotein mimic. *Biorg. Med. Chem.* *10*, 2471–2478.
- (28) Felluga, F., Fermeglia, M., Ferrone, M., Pitacco, G., Pricl, S., and Valentin, E. (2002) Computational studies on the enantioselectivity of α -chymotrypsin towards α -carbomethoxy- β -lactams. *Tetrahedron: Asymmetry.* *13*, 475–489.
- (29) Bayly, C. I., Cieplak, P., Cornell, W. D., and Kollman, P. A. (1993) A well-behaved electrostatic potential based method using charge restraints for determining atom-centered charges: the RESP model. *J. Phys. Chem.* *97*, 10269–10280.
- (30) Delley, B. (1990) An all-electron numerical method for solving the local density functional for polyatomic molecules. *J. Chem. Phys.* *92*, 508–517.
- (31) Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., and Klein, M. L. (1983) Comparison of simple potential functions for simulating liquid water. *J. Chem. Phys.* *79*, 926–935.
- (32) Darden, T., York, D., and Pedersen, L. (1993) Particle mesh Ewald: an $N^2 \log(N)$ method for computing Ewald sums. *J. Chem. Phys.* *98*, 10089–10092.
- (33) Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, A., and Haak, J. R. (1984) Molecular dynamics with coupling to an external bath. *J. Chem. Phys.* *81*, 3684–3690.
- (34) Caldwell, G., Neuse, E. W., and Perlwitz, A. G. (1997) Water soluble Polyamides as potential drug carriers. IX. Polyaspartamides grafted with amine-terminated Poly(ethylene oxide) chains. *J. Appl. Pol. Sci.* *66*, 911–919.
- (35) Higuchi, T., and Connors, K. A. (1965) Phase-solubility techniques. *Adv. Anal. Chem. Instrum.* *4*, 117–212.
- (36) Slikker, W., Jr., Lipe, G. W., and Newport, G. D. (1981). High-performance liquid chromatographic analysis of estradiol-17 β and metabolites in biological media. *J. Chromatogr. B: Biomed. Sci. Appl.* *224*, 205–219.
- (37) Gabizon, A., Horowitz, A. T., Goren, D., Tzemach, D., Mandelbaum-Shavit, F., Qazen, M. M., and Zalipsky, S. (1999) Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies. *Bioconjugate Chem.* *10*, 289–298.
- (38) Williams, R. O., III, Mahaguna, V., and Sriwongjanya, M. (1998) Characterization of an inclusion complex of cholesterol and hydroxypropyl- β -cyclodextrin. *Eur. J. Pharm. Biopharm.* *46*, 355–360.
- (39) Ooya, T., and Yui, N. (1999) Polyrotaxane: synthesis, structure, and potential in drug delivery. *Crit. Rev. Ther. Drug Carrier Syst.* *16*, 289–328.