International Journal of Pharmaceutics 423 (2012) 452–460

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Tocol modified glycol chitosan for the oral delivery of poorly soluble drugs

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ARTICLE INFO

Article history:
Received 5 October 2011
Received in revised form 5 December 2011
Accepted 8 December 2011
Available online 16 December 2011

Keywords:
Chitosan
Amphiphilic chitosan
Tocopherol
Micelles
Oral drug delivery
Poorly soluble drugs

ABSTRACT

The aim of this study was to develop tocol derivatives of chitosan able (i) to self-assemble in the gastrointestinal tract and (ii) to enhance the solubility of poorly soluble drugs. Among the derivatives synthesized, tocopherol succinate glycol chitosan (GC-TOS) conjugates spontaneously formed micelles in aqueous solution with a critical micelle concentration of 2 $\mu g\,mL^{-1}$. AFM and TEM analysis showed that spherical micelles were formed. The GC-TOS increased water solubility of 2 model class II drugs. GC-TOS loading efficiency was 2.4% (w/w) for ketoconazole and 0.14% (w/w) for itraconazole, respectively. GC-TOS was non-cytotoxic at concentrations up to 10 $mg\,mL^{-1}$. A 3.4-fold increase of the apparent permeation coefficient of ketoconazole across a Caco-2 cell monolayer was demonstrated. Tocol polymer conjugates may be promising vehicles for the oral delivery of poorly soluble drugs.

1. Introduction

Contemporary drug pipelines are becoming increasingly more complex to formulate and oral delivery of new drug entities is becoming ever more difficult since they exhibit poor water solubility, poor dissolution rate and low oral bioavailability in the gastrointestinal tract (Gaucher et al., 2010; Mathot et al., 2007; Pinnamaneni et al., 2002). This emphasizes the need of novel drug solubilization technologies for the oral administration of sparingly soluble drugs (Gaucher et al., 2010; Latere et al., 2008; Plapied et al., 2011).

Besides classical strategies like drug micronization or use of surfactants, cosolvents, complexing agents and lipids, innovative strategies using nanocarriers have been widely studied. Among these nanocarriers, polymeric micelles are of particular interest based on the functionality and utility of polymeric systems. Polymeric micelles are composed of amphiphilic block copolymers. These materials self-assemble above their critical micelle concentration (CMC) to form micelles. The hydrophilic moiety of the copolymer forms the outer layer of the micelle (the corona) while the hydrophobic block forms the core. The hydrophobic domain can interact with poorly water soluble drugs resulting in their solubilization within the micelle. Micelles show high thermodynamic stability, increase of the solubility of poorly soluble drugs and can enhance drug permeation across the physiological barriers (Ebrahi et al., 2011; Gaucher et al., 2010; Kim et al., 2006; Latere et al., 2008; Plapied et al., 2010, 2011; Torchilin, 2007). Usual issues associated with the use of these materials are related to the complex processing required for drug loading (Gaucher et al., 2010; Wiradharma et al., 2009).

A wide range of materials, such as synthetic or natural polymers, has been employed as hydrophilic moiety. Among these, chitosan (CS), a linear aminopolysaccharide composed of randomly distributed (1–4) linked D-glucosamine and *N*-acetyl-D-glucosamine units, has received increasing attention owing to its biocompatibility, non-toxicity and low-immunogenicity (Hyung et al., 2006). Moreover, its cationic properties below pH 6.5 favor mucoadhesion in the gastrointestinal tract. Hydrophobically modified chitosans have been successfully synthesized *via* derivatization of the primary hydroxyl and amine groups located on the backbone of chitosan. Nevertheless, due to the insolubility of chitosan observed at pH values above its p*K*a (6.4) in water, micelles of amphiphilic chitosan rapidly precipitate in biological solution (pH 7.4). Therefore, water-soluble chitosan derivatives have often been used for development of drug delivery systems like

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doi:10.1016/j.ijpharm.2011.12.010

glycol chitosan (GC) which exhibits good solubility over a broad range of pH (Bonferoni et al., 2009; Hyung et al., 2006; Nam et al., 2009). Amphiphilic derivatives of water soluble chitosan with lower molecular weight, chitosan oligosaccharide (CSO), have also been reported as efficient vector for drugs and siRNA at physiological pH value (7.2–7.4) (Noh et al., 2011; Zhou et al., 2010).

The aim of the study was to synthesize amphiphilic polymers made of tocol and chitosan to develop tocol derivatives of chitosan able (i) to self-assemble in the gastrointestinal tract and (ii) to enhance the solubility of poorly soluble drugs. The rationale for choosing tocols is their biocompatibility and their outstanding ability to solubilize a variety of water-insoluble compounds named tocophilic compounds (Constantinides et al., 2006). This approach contrasts with the usual methodology applied for micellar systems which consists in selecting the system with the highest drug encapsulation values after experimental tests. Hence, knowing tocophilic properties of drugs could potentially speed up the choice of the best-fitted micellar system. Two tocol compounds were chose: (i) D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) and (ii) D-α-tocopherol succinate (TOS). The TPGS was selected because of its ability to inhibit P-glycoprotein efflux. It can self-assemble in micelles but with a CMC value of 0.02 mM which is high in comparison to amphiphilic chitosan (Constantinides et al., 2006; Sadoqi et al., 2009). So it was hypothesized that the grafting of TPGS to chitosan would decrease the CMC value due to the greater thermodynamic stability of polymeric micelles (Torchilin, 2007). TOS provides the advantage of a simpler synthesis scheme compared to TPGS which needs to be activated before being grafted on the chitosan backbone. The influence of the hydrophilic moiety on the properties of the tocol derivatives of chitosan was assessed by using three derivatives of chitosan: (i) CS, (ii) GC and (iii) CSO. The physico-chemical characteristics of the obtained polymers such as size, morphology of the supramolecular structures and CMC were explored. The ability of the polymers to enhance the solubility of a tocophilic drug (itraconazole) and non-tocophilic drug (ketoconazole) was tested. Finally cytotoxicity and permeation studies were conducted using Caco-2 cells monolayer as model for intestinal barrier.

2. Materials and methods

2.1. Material

Glycolchitosan (GC) (Mw 250 kDa, deacetylation degree 0.83), chitosan oligosaccharide (CSO) (MW 5 kDa, deacetylation degree 0.90), D-α tocopherol succinate (TOS), D-α-tocopherol polyethyleneglycol 1000 succinate (TPGS), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxyl succinimide (NHS), succinic anhydride and pyrene (>99% purity) were purchased from Sigma Aldrich. Chitosan (CS) (Mw 78 kDa, degree of deacetylation 0.83) was kindly supplied by Kitozyme (Belgium). COS was purified by dialysis against MilliQ water (porosity 1 kDa) in order to remove the residual lactic acid traces before any chemical modification. All chemical reagents used for the Caco-2 culture were purchased from Invitrogen Life Technologies (Merelbeke, Belgium). Acrodisc® syringe filters for aqueous media with a diameter of 0.2 μm (Tuffryn® membrane), 0.45 μm (Tuffryn® membrane) and 1.2 μm (Supor® membrane) were purchased from Pall Newquay (Cornwall, U.K.). Millex®-AA syringe filters for aqueous media with a diameter of 0.8 μm (Millipore MF membrane) were purchased from Millipore (France). All other chemicals were of analytical grade and used as received.

2.2. Synthesis of the polymers

The tocol derivatives of chitosan were synthesized by the formation of an amide bond between the primary amine groups of chitosan and carboxylic acid functions of tocols as previously reported for other amphiphilic polymers (Kwon et al., 2003). The reaction occurs in aqueous/methanol medium in the presence of EDC and NHS. Prior to the coupling, TPGS was functionalized with a carboxylic acid group (Fig. 1). Briefly, 1.25 g of TPGS, 0.16 g of succinic anhydride and 200 μL of triethylamine were stirred 24 h in 50 mL of dichloromethane at 30 °C under strict anhydrous conditions. The reaction mixture was washed three times with a NaCl. The organic phase was dried over Na_2SO_4, filtered and the solvent was evaporated under vacuum. The activation of TPGS was confirmed by ^{1}H NMR analysis (data not shown) and the yield of 97%.

Six derivatives were synthesized: (i) TPGS derivative of CS (CS-TPGS), (ii) TOS derivative of CS (CS-TOS), (iii) TPGS derivative of GC (GC-TPGS), (iv) TOS derivative of GC (GC-TOS), (v) TPGS derivative of CSO (CSO-TPGS) with a substitution degree of 10% (mol/mol) and (vi) CSO-TPGS with a substitution degree of 20% (mol/mol) (Fig. 2). Substitution degree is approximatively 3 times higher in the case of the TPGS derivatives to get the same quantity of hydropbic moiety on the derivatives.

CS, GC and CSO were first dissolved 24 h with stirring in 20 mL of water (in 1% (v/v) acetic solution (pH 4) for CS) before adding EDC. TOS and NHS were dissolved in 60 mL of methanol whereas activated TGPS and NHS were dissolved in water before being added drop wise to the aqueous solution. The resulting solution was stirred for 24 h at room temperature. Then, the solution was dialyzed (Dialysis Tubing Thermo Scientific SnakeSkin, MWCO 12 kDa) 3 times 24 h with increasing water content (1:4 (v/v) water:MeOH, 1:1 (v/v) water:MeOH; pure water at day 1, 2 and 3 respectively). The ratio between the volume of solvent outside the dialysis membrane and the volume of the solution inside it, was at least 30. Finally, the dialyzed solution was lyophilized. The lyophilisate was stored at −20 °C (Fig. 2) (Kwon et al., 2003) (Table 1).

The tocol derivatives of chitosan were thoroughly characterized by ^{1}H NMR. All spectra were recorded on a Bruker Advance II 500 MHz spectrometer. Tocol derivatives of chitosan were dissolved in a deuterated water solution (D_2O) containing deuterated trifluoroacetic acid (CF_3COD) (pD = 5.5) and all measurements were carried out at a temperature of 70 °C. The internal solvent peak (D_2O) acts as the reference (4.79 ppm). Tocol derivatives of glycol chitosan were dissolved in a deuterated mixture containing (i) $D_2O + CF_3COD$ (pH = 4) and (ii) deuterated methanol (CD_3OD) in a volume ratio of 1:3 respectively. These measurements were carried out at 25 °C and CD_3OD acts as the reference (3.31 ppm).

2.3. Critical micelle concentration

To determine if the polymers were able to self-assemble, the critical micelle concentration (CMC) was determined using the fluorescence probe method (Ould-Ouali et al., 2004). Three milliters of a pyrene stock solution (10^{-6} M) in acetone were evaporated. Then, 5 mL of an aqueous solution of the derivatives were added to the pyrene. The pyrene concentration was set to 6×10^{-7} M for all samples. The polymer concentration varied from 10^{-8} to 1 mg mL^{-1}. Solutions were stirred during 36 h before recording the spectrum at room temperature. Fluorescence spectra were recorded with a Varian Cary Eclipse Fluorescence spectrophotometer (Agilent Technologies, Diegem, BE). The excitation wavelength (λ_{ex}) was 366 nm. The selected emission wavelengths (λ_{em}) were those corresponding to the maximum intensities of the first (I_1; λ_{em} = 373 nm) and the third peak (I_3; λ_{em} = 383 nm) in the emission spectrum. To analyze the microenvironment and the CMC of the derivatives, the

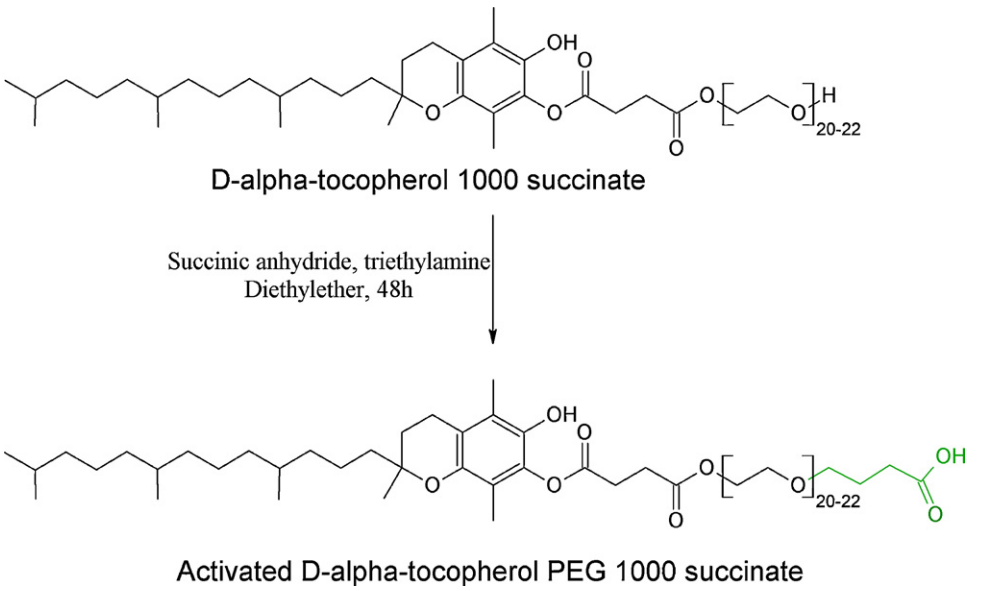

Fig. 1. Activation of the D-alpha-tocopherol PEG1000 succinate.

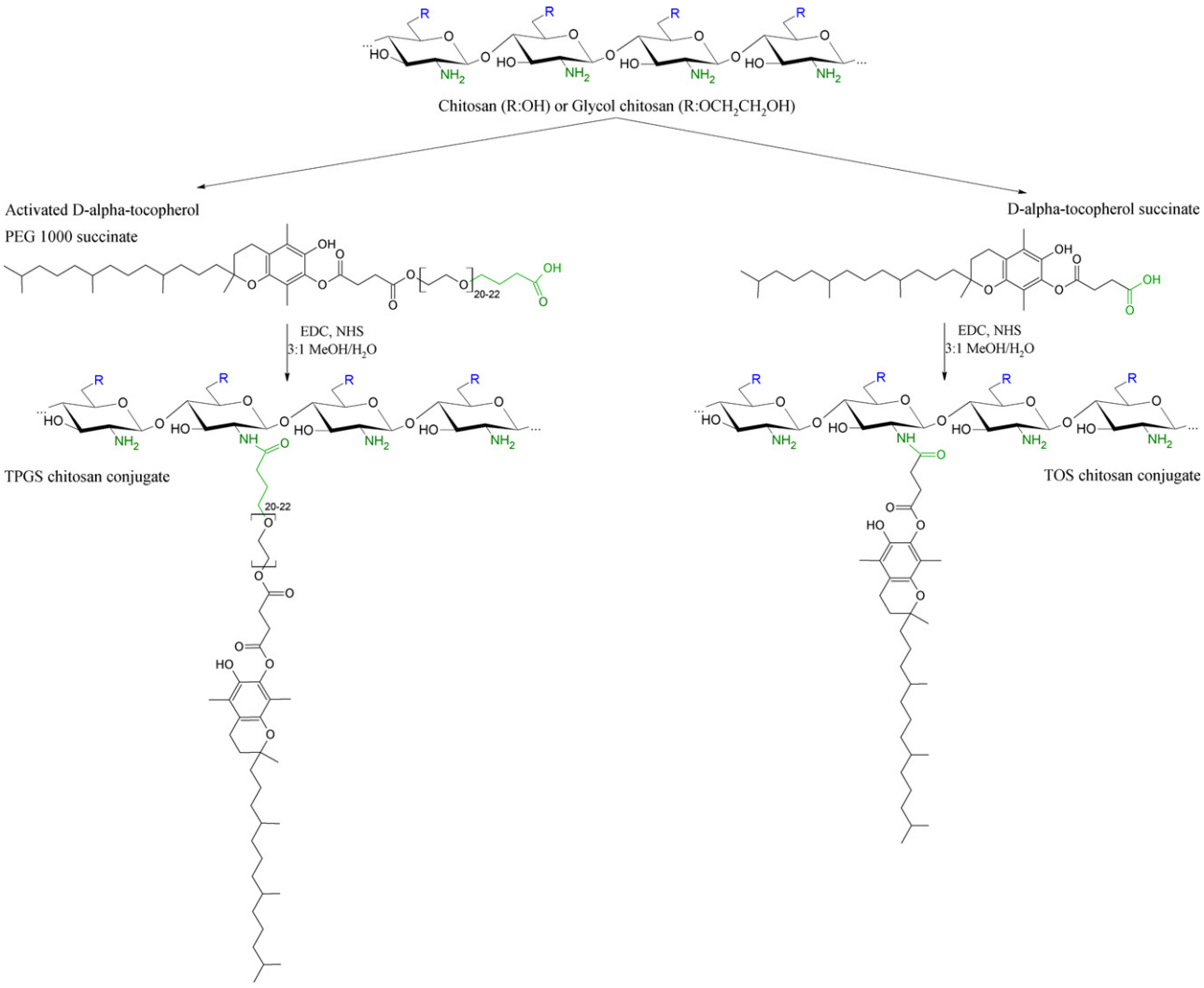

Fig. 2. Synthesis of tocol derivatives of chitosan.

Table 1
Amounts of the different chemicals involved in the synthesis of the different tocol derivatives of chitosan, their tocol substitution degree of and their behavior in aqueous solution.

Polymers	Weighted amounts (mg)				Substitution degree (%, mol/mol)	Particle formation in water
	Chitosan	Tocol	EDC	NHS		
CS-TPGS	125.0	240.3	57.0	34.3	20	Aggregation
CS-TOS	125.0	23.5	17.0	10.2	6	
GC-TPGS	125.0	191.8	45.5	27.3	20	Aggregation
GC-TOS	125.0	18.7	13.6	8.1	6	Micellization
CSO-TPGS	125.0	122.8	29.2	17.5	10	Gelation
	125.0	245.7	57.8	34.7	20	

ratio I_1/I_3 was determined in triplicate. The emission spectrum and hence the I_1/I_3 ratio of pyrene depends strongly on the polarity of the environment surrounding the probe molecules. This covers a wide range of polarity from water ($I_1/I_3 = 1.9$) to cyclohexane ($I_1/I_3 = 0.6$). This scale was used to evaluate the microenvironment in the micellar core (Nivaggioli et al., 1995). To determine the CMC, the I_1/I_3 values were averaged over 3 values plotted *versus* the polymer concentration. As the polymer concentration in the aqueous solution increased, the I_1/I_3 ratio decreased and then reached a plateau. The CMC was determined at the intersection of the 2 lines obtained by linear regression.

2.4. Size and morphology

2.4.1. Size and ζ potential

At first, the average size of a 0.01 mg mL^{-1} derivative was determined using a Multi-Angle Dynamic Light scattering instrument, Malvern CGS3 (Malvern Instruments, UK). The measurements were made at several angle (30°, 50°, 60°, 80°, 90°, 110° and 130°) with a 15 s acquisition time. The water was filtered on a 0.22 μm filter and the polymer was dissolved during 24 h under constant stirring. Polymer solutions were stabilized for 15 min at 25 °C before each analysis. Measurements were repeated 3 times. Polyelectrolyte effect was also studied by adding 2 M NaCl solutions. The hydrodynamic radius (Rh) was determined using the Stokes–Einstein relation:

$$\mathrm{Rh} = \frac{kT}{6\pi\eta D}$$

where k is the Boltzmann constant (1.381×10^{-23} J K^{-1}), T is the temperature (298.65 K), η is the fluid viscosity ($\eta_{\mathrm{water},\,20\,^\circ\mathrm{c}} = 1.002 \times 10^{-3}$ Pa s) and D is the diffusion coefficient (m^2 s^{-1}).

The average particle size and size polydispersity were also determined by photon correlation spectroscopy using a Malvern Nano ZS (Malvern Instruments, UK). Phosphate Buffer Saline (PBS) (pH 7) and HCl 0.1 M medium (pH 1) were tested. For each solution analysis, the media was filtered on a 0.22 μm filter and the polymer was dissolved during 24 h under constant stirring. The final solution was filtered through a 0.8 μm PVDF filter. Zeta potential of the derivatives was measured in KCl 1 mM with a Malvern Nano ZS (Malvern Instruments, UK) at 25 °C. The instrument was calibrated with reference polystyrene nanoparticles (Malvern Instruments, UK).

2.4.2. Atomic force microscopy (AFM)

A 0.05 mg mL^{-1} polymer solution was spin-coated on silicium substrate at 4000 rpm during 30 s. AFM images were obtained using an intermittent contact (tapping) mode Nanoscope IV (Veeco Instruments Inc, NY, USA). NCI tips were used with a resonance frequency of 330 kHz and a constant stiffness of 48 N m^{-1}.

2.4.3. Transmission electron microscopy (TEM)

TEM was performed on a LEO 922 microscope, operating at 200 kV accelerating voltage in bright field mode. The images were formed by unscattered electrons only. Samples for TEM experiments were prepared by drop-casting of the derivative solution (0.05 mg mL^{-1}) onto a carbon-coated TEM grid. Finally, the samples were exposed to RuO_4 vapors during several minutes before measurement.

2.5. Drug encapsulation

The drug solubility in the micelles was measured using the filtration method. First, a polymer solution in Hank's balanced salt solution (HBSS) was prepared by stirring for 24 h at a concentration of 0.2% (w/v). An ethanolic drug solution was then added to the aqueous polymer solution. The open vials containing the drug/polymer solution were stirred for 24 h to allow evaporation of ethanol. The drug was always in excess. The suspension was then filtered through a 1.2 μm PVDF filter to remove undesirable drug. The filtered solution was immediately diluted in ethanol. The aqueous solubility of the drug was measured by mixing an excess of the drug for 24 h and finally by filtering the solution through a 0.45 μm and a 1.2 μm PVDF filter.

The quantification of the drug was based on an HPLC method. The drug loading content (LC, %, w/w) of the micelles was calculated with the following equation:

$$\mathrm{LC}(\%,\,\mathrm{w/w}) = \frac{\text{Amount of drug in the micelles}}{\text{Amount of polymer}} \times 100$$

2.6. In vitro drug release

The *in vitro* release of ketoconazole from GC-TOS micelles was evaluated by a dialysis method. Ketoconazole loaded micelles (ketoconazole = 245 μg mL^{-1}, 1% polymer) were prepared in PBS. 4 mL was placed into a dialysis membrane tubing (Spectra/Por 7, regenerated cellulose, Spectrum Laboratories) with a 2000 Da molecular weight cut-off and immersed into 50 mL PBS at 37 °C with stirring. Dialysis was performed for 48 h and the amount of ketoconazole released into the medium was measured after 30 min, 1, 2, 6, 12, 24 and 48 h. The ketoconazole concentration in the different samples was determined by HPLC (Danhier et al., 2009; Saxena et al., 2011; Tiwari et al., 2009).

2.7. HPLC analysis

The concentrations of ketoconazole and itraconazole were determined at 25 °C by reversed-phase high performance liquid chromatography (Agilent 1100 series, Agilent Technologies, Diegem, BE) with a 125/4 Nucleodur RP C18 column 5 μm. The injection volume was 50 μL in both cases. The other parameters are summarized in Table 2 (Kovacs et al., 2009; Mellaerts et al.,

Table 2
HPLC parameters used for the drug detection ($n = 3$).

HPLC parameters	Itraconazole	Ketoconazole
λ (nm)	260	206
Flow rate (mL min^{-1})	1.5	1
Linearity range (μg mL^{-1})	0.5–50	0.5–50
R^2	0.991	0.999
Limit of detection (μg mL^{-1})	0.1	0.2
Interassay variance	<4%	<3%
Mobile phase	Acetonitrile and tetrabutyl ammonium hydrogen sulfate 0.01 N (55:45, v/v)	Methanol and ammonium acetate 0.5% (80:20, v/v)

2010). The methods were validated and no solvent interference was observed.

2.8. Caco-2 cells experiments

Caco-2 cells were obtained from the American Type Culture Collection (ATCC). The cultures were mycoplasma free (mycoplasma detection kit; Roche GmbH, DE). Caco-2 cells were routinely maintained in plastic culture flasks (162 cm^2) (Corning Incorporated, NY, USA) with 0.2 μm vent cap. Caco-2 cells were harvested before reaching confluence with 0.25% (w/v) trypsin and 0.2% (w/v) EDTA 15 min at 37 °C. The culture medium, Dulbecco's Modified Eagle Medium (DMEM), was supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 10% fetal bovine serum (FBS).

2.8.1. Cytotoxicity assays

For the cytotoxicity assays, cells were used between passage 9 and 29. Cells were seeded in 96-well plates at the density of 20,000 cells per well. The seeding was performed with 100 μL of the supplemented DMEM culture medium. Cells were incubated overnight at 37 °C under 10% CO_2. The culture medium was then replaced with 100 μL of polymer solutions (from 0.05 mg mL^{-1} to 10 mg mL^{-1} in HBSS). Triton X-100 (1%, w/v) was used as positive control while HBSS was used as the negative control. The cells were incubated 120 min. After removal the solutions, 100 μL of culture medium containing 10 μL of a 5 mg mL^{-1} 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, Bornem, Belgium) stock solution was added to each well. Cells were again incubated for 180 min. The media was removed from all wells and 100 μL of DMSO (Merck, Germany) was added to dissolve the formazan by-product. Absorbance of the 96-well plates was assessed with a Multistkan EX (Thermo Fisher Scientific Corporation, USA) at 620 nm. From the absorbance, the viability of the cells expressed in percentage was calculated using the negative control as reference (100% of viability) (Mathot et al., 2007; Ould-Ouali et al., 2004).

2.8.2. Permeation studies

The Caco-2 cells were used between passage 24 and 29 for the permeation studies. Caco-2 monolayers were grown in 12 well inserts containing a track-etched polyethylene terephthalate 1 μm microporous (BD Biosciences, USA) for a period of 21 days, at a seeding density of 1.8×10^5 cells cm^{-2}. The culture medium was exchanged every two days for 21 days.

The cell monolayers were washed with HBSS supplemented with FBS 1% (v/v) (0.5 mL in the apical compartment and 1.5 mL in the basolateral side) twice within 30 min at 37 °C. For the apical to basolateral transport, the medium was removed from the upper compartment and replaced with 500 μL of HBSS containing 1% (w/v) ketoconazole and/or GC-TOS on the apical side. The monolayers were incubated for 90 min at 37 °C. The amount of drug crossing the monolayer was assayed by removing of the basolateral compartments 1.5 mL after 120 min. The amount of drug crossing the Caco-2 monolayer was determined by HPLC. The transport experiments were performed at least in triplicate (Mathot et al., 2006, 2007). The drug apparent permeability coefficient (P_{app}, cm s^{-1}) was calculated using the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} \times \frac{1}{C_i A}$$

where $\Delta Q/\Delta t$ is the amount of drug (Q) appearing per time unit (t) in the acceptor compartment, C_i is the initial concentration in the apical compartment (μg mL^{-1}), A is the surface area of the Caco-2 monolayer ($A = 0.9$ cm^2) (Mathot et al., 2006; Ould-Ouali et al., 2004).

Transepithelial electrical resistance (TEER) was measured using a Voltohmmeter EVOM (World Precision Instrument, Hertfordshire, England). TEER of the cell monolayers was controlled 30 min before starting the experiment and at the end of the experiment. Only monolayers with initial values higher than 400 Ω cm^2 were used (Ould-Ouali et al., 2004). The resistance of HBSS alone (8 Ω cm^2) was considered as background resistance and subtracted from each TEER value (Mathot et al., 2007).

Lactate dehydrogenase (LDH), a cytosolic enzyme that is readily released upon cell membrane damage, was used to evaluate cellular toxicity. LDH was dosed in apical compartments to evaluate formulation cytotoxicity using lactate dehydrogenase kit (Roche, NL). Apical media were collected and centrifuged (250 g, 5 min). LDH was quantified according to manufacturer's instructions (Plapied et al., 2010).

2.9. Statistics

Statistical analyses were completed using JMP software (SAS Institute Inc., version 4.0.2). LDH, TEER and P_{app} experiments were compared using a t-test (Mann–Whitney test). Values of $p < 0.05$ and $p < 0.001$ were considered statistically significant and highly significant, respectively.

3. Results and discussion

3.1. Polymer synthesis and characterization

Several tocol derivatives of CS, COS and GC were synthesized through the reaction of the carboxyl groups of TOS or activated TPGS and the amino groups of CS, COS and GC (Figs. 1 and 2). GC was used to increase nanoparticle stability at pH 7 since it has higher water solubility than chitosan. COS was used for a lower MW of the hydrophilic moiety. Successful syntheses of tocol derivatives were confirmed by ^{1}H NMR spectrometry as illustrated for GC-TOS in Fig. 3 (Kwon et al., 2003; Noh et al., 2011). The appearance, after reaction, of the characteristic peaks of the aliphatic chain of the tocopherol moiety demonstrates clearly its incorporation onto the GC. Interestingly, the spectra of GC and of GC-TOS in D_2O are identical and the addition of a significant amount of CD_3OD (75 v-%) to the medium is necessary to bring up the signals from the TOS moieties. This is explained by the micellization of the amphiphilic GC-TOS in water (see infra), leading to a shielding of the proton signal of the micellar core (TOS) by the surrounding GC corona. Adding CD_3OD, a good solvent for TOS, helped its solubilization in the medium and cancelled, in that manner, the shielding effect occurring in micelles (Kwon et al., 2003).

3.2. Selection of self-assembling chitosan tocopherol derivatives

We conducted a preliminary test by dynamic light scattering to check if the synthesized tocol derivatives of chitosan were able

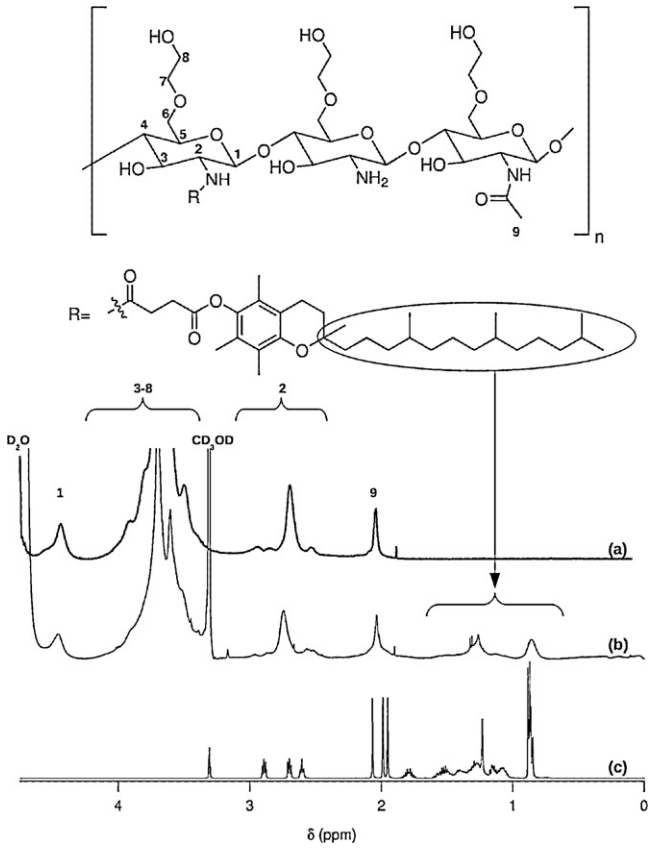

Fig. 3. ^{1}H NMR spectra of GC in D_2O (a), of GC-TOS in D_2O/CD_3OD (1:3, v/v) (b) and of TOS in CD_3OD (c) at 25 °C.

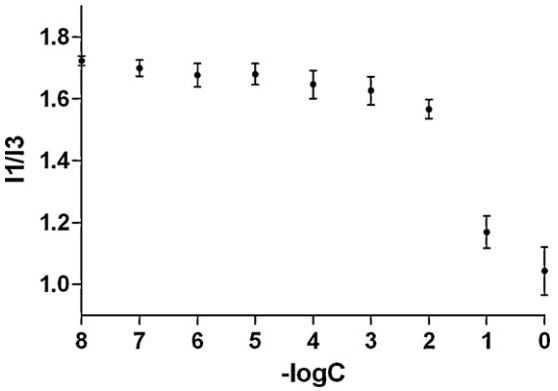

Fig. 4. I_1/I_3 ratio *versus* polymer concentration of GC-TOS ($-\log C$) in water ($n = 3$).

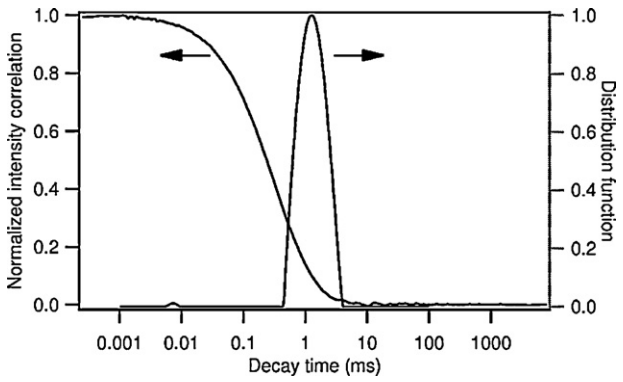

Fig. 5. Intensity auto-correlation function (left) and Contin analysis of the DLS data acquired at 90° for GC-TOS in water (0.01 mg mL^{-1}).

to spontaneously self-assemble in aqueous solutions and to select polymers with potential nanocarrier properties of poorly soluble drugs.

Among the TOS modified polymers, only the hydrophobically modified glycol chitosan with α-tocopherol succinate (GC-TOS) with a DS of 6% (mol/mol) formed nanosized self-assembling particles in phosphate buffer at pH 7 and HBSS. 20% tocopherol substitution levels of GC-TOS led to the formation of large aggregates (data not shown).

Solubilization of CS-TOS was not possible at pH 7 due to the low solubility of chitosan under these conditions.

CS-TPGS and GC-TPGS formed jelly micronic structures and thus were not appropriate as micellar vehicle. Interestingly, the CSO-TPGS derivatives formed gels in acid medium (Fig. 6C).

In conclusion, only GC-TOS (DS 6%) self-assembles in nanosized particles. Hence, we focused our research on the GC-TOS amphiphilic derivative.

3.3. Physicochemical properties of GC-TOS

To check whether amphiphilic GC-TOS self-assemble in micelles, CMC, size and morphology of the GC-TOS particles were investigated.

3.3.1. Critical micelle concentration

The CMC of GC-TOS was determined by the fluorescent probe method. When the concentration of GC-TOS was increased, shifts occurred in the emission spectra (I_1: λ_{em} from 372 to 374 nm and I_3; λ_{em} from 383 to 385 nm) of pyrene resulting from a shift from an aqueous polar environment to a more hydrophobic medium (Nivaggioli et al., 1995). The CMC of GC-TOS was determined to be 2 ± 2 μg mL^{-1} (Fig. 4). As expected, the micelle core was very hydrophobic ($I_1/I_3 \approx 1.05$).

Results showed that GC-TOS has a very low CMC value. Similar results were reported for tocopheryl succinate COS (CMC 4 μg mL^{-1}) (Noh et al., 2011). In comparison, other amphiphilic chitosan like 5-β cholanic grafted GC and *N*,*N*-diethylnicotinamide oligomers grafted GC exhibited higher CMC values, respectively 47 μg mL^{-1} and 41 μg mL^{-1}(Kwon et al., 2003; Saravanakumar et al., 2009). These results could be related to the π–π interactions induced by the aromatic rings of tocopherol which have been identified as a CMC lowering factor (Kwon et al., 2003; Wiradharma et al., 2009). The CMC of GC-TOS is sufficiently high to withstand dilution and ensure the delivery of a carried drug to its absorption site (Gaucher et al., 2010). Consequently, GC-TOS displays a sufficient thermodynamic stability for oral administration of poorly soluble drugs.

3.3.2. Size and morphology

The size and the morphology of micellar objects in water were investigated by multi-angle DLS. The amphiphilic GC-TOS derivative formed nanoparticles spontaneously in aqueous solution (PBS or distilled water). The formation of nanoobjects has been demonstrated as evidenced in Fig. 5 which shows the relaxation curve measured at an angle of 90° (intensity auto-correlation function) and the corresponding Contin analysis of GC-TOS in water. A hydrodynamic radius of 101 nm for GC-TOS particles was obtained in these conditions. The measurements at several angles showed no angular dependence of the hydrodynamic radius, suggesting the formation of spherical objects.

These results were confirmed by AFM and TEM analysis which both showed nanoparticles with a diameter of about 90 nm and with a spherical shape (Fig. 6A and B).

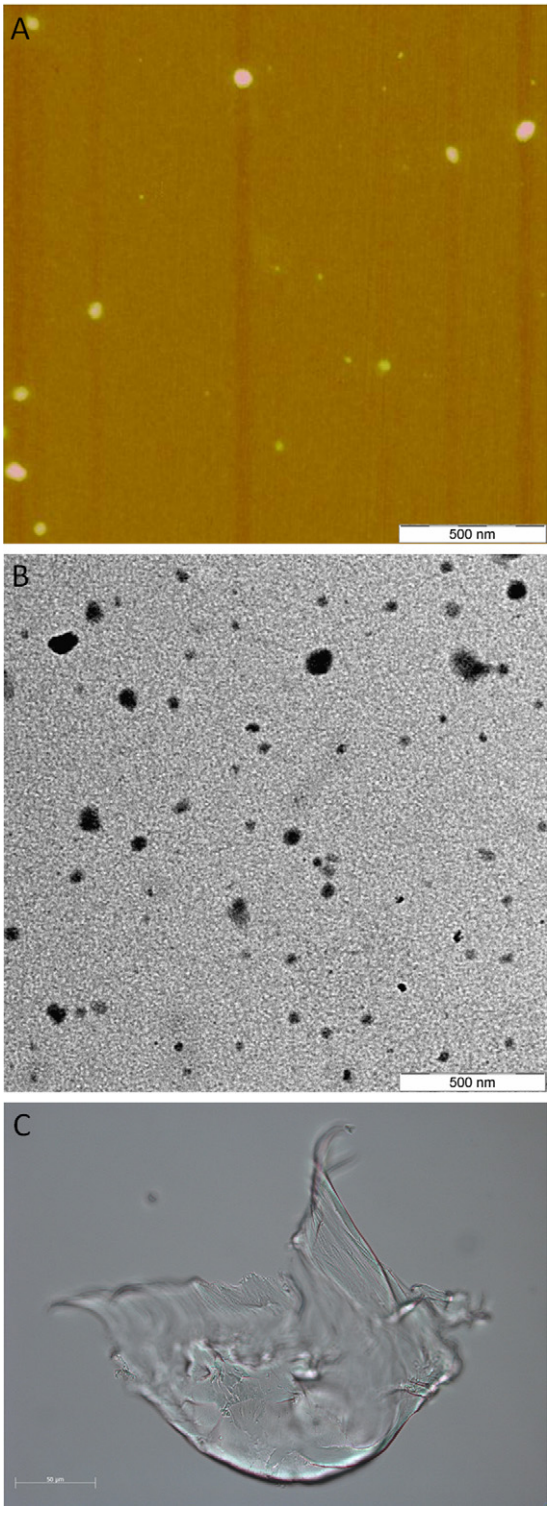

Fig. 6. AFM (A) and TEM (B) images of GC-TOS aqueous solution and (C) of GC-TPGS jellyfish micronic particle.

By increasing the concentration of GC-TOS till 1 mg mL^{-1}, micellar aggregates were observed as evidenced by DLS. The Rh was equal to 283 ± 30 nm (PDI: 0.4 ± 0.3) in PBS (pH 7) and 353 ± 25 nm (PDI: 0.4 ± 0.3) in HCl 0.1 N. The ζ potential of the aggregates was 8.6 ± 0.5 mV indicating free amino groups positively charged on the particle surface (n = 3). The presence of such aggregates, a well-known problem for nanoparticles in solution, could be explained by the high molecular weight of the GC (~250 kDa). Several studies have already highlighted the size and structure dependency of self-assembled particles to the molecular weight of chitosan derivatives and more recently for tocopherol COS derivatives (Kim et al., 2001; Noh et al., 2011; Wang et al., 2001). It has been suggested that increasing the micelle concentration of CS-stearic derivative with a molecular weight higher than 40 kDa induced further aggregation into a poorly organized birdnest-like structure that was assigned to the higher rigidity of the chitosan chain (Kim et al., 2001). It could be hypothesized that GC-TOS might also form bird-nest like structures, at increasing GC-TOS concentration due to inter- and intra-chains interactions with particles containing several hydrophobic domains. Several small GC-TOS particles could aggregate in larger structures when increasing polymer concentration. The observation of a few large aggregates by TEM and AFM pictures supports this second hypothesis (Fig. 6). As GC-TOS are intended for oral delivery, the large aggregates in concentrated GC-TOS solutions were however not regarded as a limitation. Lowering the molecular weight of GC appears to be a valuable approach for further investigations. However, organic solvent and a dialysis step is required for the formulation of COS with tocopherol at DS of 10 and 20%, limiting their use for self-assembling system in the gastrointestinal tract (Noh et al., 2011). In addition, quaternization of some of the amino groups of low-molecular weight GC could be another interesting way to force repulsion between particles and to avoid further aggregation of the GC-TOS nanoparticles.

Table 3
Solubility (μg mL^{-1}) of ketoconazole and itraconazole in phosphate buffer (pH 7) GC-TOS solutions, measured after filtration through a 1.2 μm PVDF filter, and drug loading efficiency (LE) of the polymer which is obtained by dividing the drug solubility in the GC-TOS solution and the mass of GC-TOS (n = 3).

Solubility characteristics of drugs	GC-TOS solutions	
	0.2 (%, w/v)	1 (%, w/v)
Ketoconazole		
Solubility (μg mL^{-1})	41 ± 8	245 ± 16
LE (%, w/w)	2.0 ± 0.4	2.4 ± 0.2
Itraconazole		
Solubility (μg mL^{-1})	2.8 ± 0.2	14 ± 1
LE (%, w/w)	0.14 ± 0.01	0.14 ± 0.01

3.4. Drug encapsulation

One of the key features of polymeric micelles is their capacity to solubilize hydrophobic drugs into their core. Hence, the loading efficiency and the solubility of two poorly soluble drugs (BCS class II) ketoconazole and itraconazole in GC-TOS solutions were investigated. These drugs were selected regarding to their tocophilic properties. Itraconazole is a tocophilic drug with an estimated vitamin E solubility of 60 mg mL^{-1} whereas ketoconazole is not tocophilic (Constantinides et al., 2006). The effect of GC-TOS concentration was also studied.

Classical methods to study drug solubilization properties of micelles could not be used. Indeed, GC-TOS was insoluble in organic solvents and the micelle sizes of GC-TOS solutions were too high to be filtered on a 0.45 μm PVDF filter (Torchilin, 2007; Ould-Ouali et al., 2004) Thus we studied drug solubility in phosphate buffer solutions (pH 7) after filtration through 1.2 μm.

The solubility of ketoconazole and itraconazole and in GC-TOS solutions, measured after filtration through 1.2 μm, are shown in Table 3. Solubility of ketoconazole, in PBS (pH 7.4) measured after filtration through 0.45 μm and 1.2 μm PVDF filters, was respectively 0.53 ± 0.05 μg mL^{-1} and 9 ± 1 μg mL^{-1}. Solubility of itraconazole could not be determined since the values were below the limit of detection of the HPLC method. According to the literature, itraconazole should have an aqueous solubility of 1 ng mL^{-1} at neutral pH and of 4 μg mL^{-1} at pH 1 (Mellaerts et al., 2010).

Solubility of ketoconazole was 41 μg mL^{-1} and 245 μg mL^{-1} for 0.2% and 1% (w/v) GC-TOS solutions respectively indicating that GC-TOS increased ketoconazole solubility by 4.5 and 27 times respectively compared to the free drug solubility measured in the same conditions. The loading efficiency of both 0.2% (w/v) and 1% (w/v) content were similar (~2.2%) since the increase of the polymer concentration induced a similar increase of the encapsulation of the drug.

Considering the limit of detection as an approximation of the water solubility of itraconazole (0.1 μg mL^{-1}), solubility of itraconazole in GC-TOS solution of 0.2% (w/v) and 1% (w/v) were increased at least by 28 times and 140 times respectively, more likely 3 orders of magnitude in regard to the predicted solubility in water (1 ng mL^{-1}, pH 7) (Kovacs et al., 2009; Mellaerts et al., 2010). The maximal loading efficiency was 0.14% (w/w) in both cases. The higher increase in solubilization is consistent with the higher tocophilic behavior of itraconazole in comparison with ketoconazole which is a non-tocophilic drug.

GC-TOS was able to increase the solubility of both ketoconazole and itraconazole but the loading efficiency strongly depended on the drug. The highest loading achieved by using the 1% (w/v) GC-TOS solution was obtained for ketoconazole (2.4%, w/w) and was 17 times higher than for itraconazole (0.14%, w/w). The loading efficiency of the two drugs was constant for the polymer concentration studied indicating drug saturation in the hydrophobic core.

Several factors could influence drug encapsulation. The hydrophobic core of the micelles (I_1/I_3 pyrene ≈ 1.05) is favorable to hydrophobic drug encapsulation. However, encapsulation of itraconazole was low in comparison to ketoconazole despite its tocophily. Indeed, 10 mg of GC-TOS contain 0.11 mL of vitamin E and should solubilize at least 65 μg of itraconazole, a value 4.6 greater than that found (14 μg), suggesting that all the tocopherol is not available for drug solubilization (Constantinides et al., 2006). Moreover, the molecular weight of the GC chain could be too high or the DS too low for a high encapsulation of the hydrophobic model drugs. It has been shown that supersaturated itraconazole solutions provoke the formation of nanofibers with length increasing up to several micrometers over the time in simulated gastrointestinal fluids (Mellaerts et al., 2010). Computer simulations of drug encapsulation in quaternary ammonium palmitoyl glycol chitosan (GCPQ) micelles have shown that larger micelles displayed a lower encapsulation efficiency when compared with smaller micelles (Ahmad et al., 2010). This could be consistent with our results since the size of GC-TOS particles is higher than GCPQ (20–30 nm) (Qu et al., 2006).

3.5. In vitro drug release

The release profile of ketoconazole from ketoconazole loaded GC-TOS micelles in PBS (pH 7.4, 37 °C) was studied. The micelles exhibited a sustained release profile up to 48 h (Fig. 7). Over the first 12 h, 47% of loaded ketoconazole was released. The micelles released 67% of ketoconazole after 24 h and 84% after 48 h. Similar sustained release profile of drugs from amphiphilic chitosan particles was already shown in previous studies, especially with glycol chitosan-5β cholanic acid (Min et al., 2008).

3.6. Caco-2 experiments

Drug delivery vehicles must be biocompatible to be successfully exploited in the pharmaceutical field. Hence, cytotoxicity of GC-TOS was measured in a Caco-2 cells monolayers, which remain the gold standard as enterocytes model, using (i) the MTT colorimetric test to assess viability, (ii) the measurement of TEER values to assess tight junction integrity, and (iii) the LDH release to evaluate the cell membrane damages. In parallel, ketoconazole transport with and without GC-TOS was performed to evaluate the influence of the polymer on the intestinal permeability of the drug (Table 4).

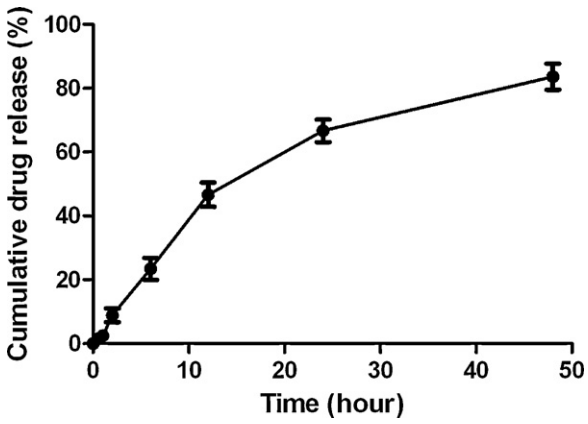

Fig. 7. *In vitro* ketoconazole release from ketoconazole loaded GC-TOS micelles in PBS (pH 7.4, 37 °C). The results were expressed as mean values ± standard deviation of 3 measurements.

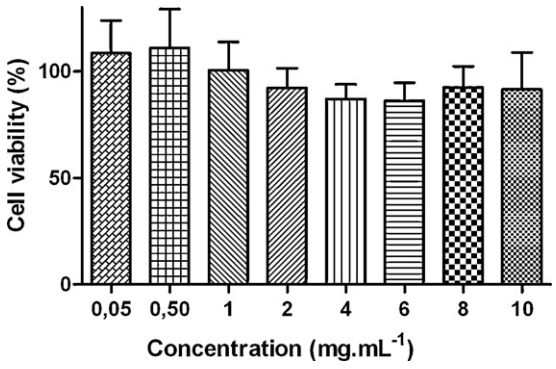

Fig. 8. Caco-2 cells viability resulting with GC-TOS solutions of various concentration (n = 5).

Based on the MTT cytotoxicity test, GC-TOS showed no cytotoxicity at concentration varying from 0.05 mg mL^{-1} to 10 mg mL^{-1} (Fig. 8). These results are consistent with previous studies which have shown the low cytotoxicity of other amphiphilic chitosan derivatives (Noh et al., 2011; Saravanakumar et al., 2009).

LDH release was less than 4% when Caco-2 monolayers were incubated with free ketoconazole (245 μg mL^{-1}), GC-TOS (1%, w/v) and GC-TOS encapsulating ketoconazole, confirming the lack of toxicity of GC-TOS. The TEER measurements at the beginning and at the end of the transport assay showed a reduction of 23% of the initial value after 2 h of incubation with 1% (w/v) GC-TOS ($p < 0.05$ *vs* HBSS) probably due to the ability of GC-TOS to open tight junctions (Smith et al., 2004).

To check if drug absorption through Caco-2 cell monolayer is enhanced when the drug is solubilized in GC-TOS micelles, ketoconazole was solubilized to its saturation solubility in 1% (w/v) GC-TOS, 245 μg mL^{-1} (Table 3) and compared with a suspension of ketoconazole at the same concentration. After 2 h of incubation in Caco-2 cell monolayers, the drug apparent permeability values (P_{app}) were determined. P_{app} of ketoconazole was 1.3×10^{-6} cm s^{-1} without the GC-TOS and 4.4×10^{-6} cm s^{-1} with GC-TOS. Thus a 3.4 fold-increase of ketoconazole absorption was observed in presence of GC-TOS (1%, w/v) and was statistically different from values determined without GC-TOS ($p < 0.05$).

The ability of chitosan to open tight junctions is one of its well-known properties (Smith et al., 2004). However, due to the size of the micelles, it is unlikely that may significantly participate to the

Table 4
Influence of a 1% (w/v) GC-TOS solution on the transepithelial electrical resistance (TEER) ($n = 3$), the LDH release ($n = 3$) and the apparent permeability (P_{app}) of ketoconazole ($n = 3$) in a Caco-2 cell monolayer.

	TEER variation (%)	LDH (%)	P_{app} ($cm\,s^{-1}$)
Ketoconazole	93 ± 6	2.9 ± 0.4	$1.3 \times 10^{-6} \pm 0.2 \times 10^{-6}$
GC-TOS	74 ± 8	3.2 ± 0.2	–
GC-TOS with ketoconazole	72 ± 7	3.9 ± 0.6	$4.4 \times 10^{-6} \pm 0.8 \times 10^{-6}$
HBSS	95 ± 5	2.8 ± 0.5	–

increase in drug absorption. The exact mechanism of the absorption enhancement was not determined but may be related to the enhancement of the passive diffusion and/or endocytosis of drug loaded micelles (Mathot et al., 2007).

4. Conclusion

Several tocol derivatives of chitosan were successfully synthesized *via* a simple peptidic-like reaction. Thanks to its amphiphilic property, tocopherol succinate glycolchitosan derivative self-assembles in water at very low polymer concentrations ensuring that its concentration in the gastro-intestinal tract will remain above the CMC after oral administration. GC-TOS increased the solubility of poorly soluble drugs and enhanced the intestinal permeation of a BCC class II drug *in vitro* with no toxicity.

Tocol derivatives of chitosan seem to be a promising and safe vehicle for the solubilization of poorly soluble drugs. Further investigations need to be conducted on the influence of the chitosan molecular weight on the self-assembly, the solubilization properties and oral drug absorption.

Acknowledgments

The authors express their acknowledgements to the Région wallonne for financial support and fellowships to Nicolas Duhem, Pierre Guillet and Jean-Marc Schumers in the frame of the Winnomat 2 CHITOPOL project. The authors also wish to Kitozyme for the supply of chitosan.

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