Physicochemical and antimicrobial properties of boron-complexed polyglycerol–chitosan dendrimers

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Abstract—A polyglycerol with dendritic structure (PGLD) was synthesized by ring-opening polymerization of deprotonated glycidol using a polyglycerol as core functionality in a step-growth process. Then, PGLD reacted with O-carboxymethylated chitosan to obtain PGLD-chitosan dendrimer (PGLD-Ch). After the reaction of PGLD-Ch with boric acid, there was a marked increase in the bulk viscosity evidencing physically that boron can initiate a charge transfer complex formation, (PGLD-Ch)B. Gel permeation chromatography analysis was used to characterize the molecular weight and the polydispersivity of the synthesized PGLD-Ch. A dendritic structure with a molecular mass of 16.7 kDa and a narrow polydispersity ($M_w/M_n = 1.05$) was obtained. ^{1}H-NMR and ^{13}C-NMR measurements were employed to assess the degree of branching in PGLD. The obtained value of 0.85 indicates the tendency toward a dentritic structure for PGLD. The glass transition temperature values of (PGLD-Ch)B membranes containing 10% and 30% PGLD were −19°C and −26°C, respectively, which favor its potential use as surface coating of several polymers. The *in vitro* cytotoxicity was evaluated using the minimum essential medium elution test assay. Extracts of boron-complexed PGLD exhibited lower cytotoxicity than the controls, suggesting that the material has an improved biocompatibility. Antibacterial studies of (PGLD-Ch)B against *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed a significant activity. Our study confirms and supports the effectiveness of (PGLD-Ch)B as an antimicrobial coating due to its capacity in suppressing the bacterial proliferation. The best *in vivo* response was found for (PGLD-Ch)B-30 membranes, which exhibited higher synthesis of collagen fibers than PGLD-ChB-10.

Key words: Polyglycerol; chitosan; dendrimers; antimicrobial properties; wound healing.

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INTRODUCTION

These days, synthetic polymer biomaterials are extensively employed in the manufacture of medical devices and implants. Medical devices range from easily inserted and retrieved contact lenses, venous catheters to surgically implanted heart valves, hip joints, coronary stents, cardiac pacemakers and artificial organs [1–3].

Infection is one of the most serious complications following implantation of a prosthetic material. The main problem in the treatment of implant-associated infection is the persistence of surface adhering microorganisms and biofilm formation due to a markedly reduced bactericidal activity of granulocytes in the vicinity of the synthetic surface [4].

It is well known that the implantation of foreign materials constitutes a risk of introduction of endogenous bacteria from the patient's own skin. The most serious problem usually encountered in the field of implanted biomaterials is the infection usually caused by staphylococci, mostly *Staphylococcus epidermidis* and *Staphylococcus aureus*, resulting in morbidity and considerable mortality indices [5–8].

The physical or chemical modification of polymer surfaces to achieve antimicrobial properties has claimed the attention of a great number of scientific research groups [9–12]. Polymer surfaces can be modified by gamma radiation (^{60}Co) or glow discharge techniques through the introduction of functional groups with an intrinsic antimicrobial activity. Another approach is the polymers' pendant functional groups that can be activated for the immobilization of bioactive drugs with antimicrobial properties.

Recently, some interesting physicochemical properties of dendrimer analogues to proteins and their high degree of surface functionality offer some attractive possibilities in biomedicine [13–19]. First, they have controllable sizes ranging from a few nanometers up to micrometers, which places them at dimensions that are smaller than or comparable to those of a cell (10–100 μm), a virus (20–450 μm), a protein (5–50 μm) or a gene (10–100 nm). Second, due to the high density of functional groups at their surface, dendrimers open up many applications involving the transport or the anchoring of bioactive substances to deliver to a targeted region of the body.

Recently, our attention has been directed to polyglycerol polymers and their derivatization with chitosan. Polyglycerol (PGL) is a fully water-soluble polymer that contains both pendant hydroxyl groups and ether linkages. The low cytotoxicity and the FDA approval of PGL as emulsifiers in the pharmaceutical and food industries make it a promising polymer for use in the biomedical field [20–23].

Dendritic polyglycerols with low glass transition temperature (T_g) are interesting polymers due to the high density of local hydroxyl groups. These functional groups may be modified in a second synthetic step and used as sites for immobilization of bioactive compounds. In this manner it is possible to tailor bioactive materials suitable for coating of implantable biomaterials devices.

Chitosan (Ch) is a polysaccharide mainly composed of D-glucosamine repeating units and a small amount of N-acetyl-D-glucosamine residues (<20%). Several interesting biological properties have been reported for chitosan, such as wound healing, immunological activity and antibacterial effects [24–26].

Currently, polyglycerols and chitosan have received a considerable attention as coating of biomaterials due to their nontoxicity and good biological compatibility with human tissue. In this sense, PGL and Ch have become an interesting alternative for the development of antimicrobial coatings.

Boric acid has been commonly used in small quantities in cosmetic formulations as antiseptic agent. Boric acid has antiseptic and antiviral activity. Aqueous solutions of boric acid have been used as mouthwashes, eye drops, skin lotions and cosmetics.

Various boron-containing materials have been synthesized and test for antimicrobial activity. Boric acid reacts with polyhydroxyl compounds as a Lewis acid to form complex in aqueous solution to produce tetrahedral anionic complexes. In spite of the some studies about the antimicrobial behaviour of amine borate materials [27], to our knowledge, no biocompatibility and antimicrobial investigations about the borates of poly(glycerol–chitosan) dendrimers (PGLD-Ch)B have been reported to date.

In the present study we prepared PLGDs with probable antibacterial activity based on the reaction of PGLD-Ch with boric acid to produce borate complexes. The water sorption, surface properties, citotoxicity and antimicrobial activities of the resulting boron complex were tried. Moreover, the *in vivo* behavior in rats of these macromolecules is also discussed. Such studies are believed to be important in the endeavour to synthesize antimicrobial active dendrimers.

MATERIALS AND METHODS

Synthesis of (PGLD-Ch)B dendrimer

Dendritic polyglycerol (PGLD) was prepared by following the divergent synthetic methodology, because of the better control provided by this technique. Polymerizations were carried out in a batch reactor equipped with a mechanical stirrer (200 rpm) and a dosing pump under nitrogen atmosphere. Glycerol (Aldrich) was extensively dried before use. The PGL core was synthesized by glycerin etherification at 533 K and nitrogen atmosphere in the presence of NaOH as catalyst and distilling the reaction water in a Dean–Stark system. The synthesized polyether was exhaustively dried in high vacuum at 120°C for 24 h in the reaction vessel. High-performance liquid chromathography (HPLC) was used to access the molecular mass. HPLC analysis was performed on a Jasco PU-980 Intelligent HPLC pump and a Jasco RI-930 Intelligent refraction index detector. The mobile phase used was acetonitrile/water (85:15). The flow rate was maintained at 1.0 ml/min and the temperature at 30°C.
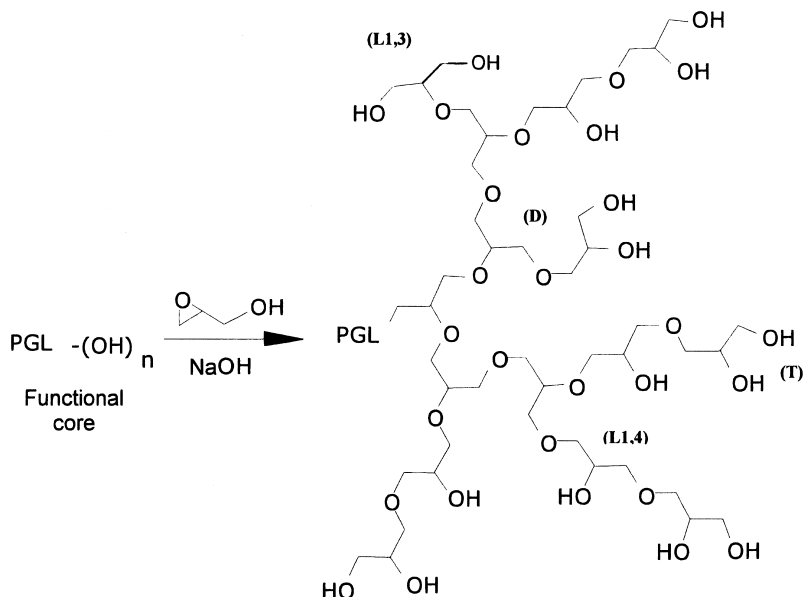
Figure 1. Schematic architecture of the polyglycerol dendrimer (PGLD) synthesized in this work. Examples of terminal (T), linear 1,3 ($L_{1,3}$) and linear 1,4 ($L_{1,4}$) units are indicated.

The synthesized PGL was partially deprotonated (15%) with potassium methylate solution (3.7 M in methanol, Fluka) by distilling off methanol from the melt. A 50-ml aliquot of glycidol (Fluka) was slowly added at 90°C over 12 h. The slow glycidol addition and the partial deprotonation allowed a better control on molecular weight and polydispersity of the dendrimer (Fig. 1). After completion of the reaction, termination was carried out by addition of a drop of acidified methanol. The PGLD dendrimer was dissolved in methanol, neutralized by filtration over cation-exchange resin and precipitated from methanol solution into diethyl ether. Then the product was purified by dialysis (benzoylated cellulose membrane with molecular mass cut-off in the range of 25 kDa, Sigma) and subsequently dried for 15 h at 80°C in vacuum. PGLD was obtained as a transparent and viscous liquid at room temperature (50 cps at 25°C).

Chitosan with a molecular mass of 150 kDa and a degree of acetylation of 15% was obtained from Fluka and was used as received. O-carboxymethylated chitosan (O-Ch) was obtained after reaction with chloroacetic acid in aqueous sodium hydroxide at 30°C overnight (yield 96%). O-Ch was esterified by refluxing it with PGLD using dimethylsulphate as catalysts under homogeneous conditions. Chitosan hydrobromide salts from the PGLD-Ch samples were prepared to assess the free amino group content by direct titration with 0.1 M NaOH solution using phenolphthalein as indicator [28, 29].

The PGLD-Ch boron complex (PGLD-Ch)B complex was performed in a glass reactor provided with stirrer, condenser and thermostatting bath. Appropriate amounts of boric acid were quantitatively introduced in the reactor containing the

PGLD-Ch. The reaction temperature was elevated to 160°C and the reactional vessel was purged with gaseous nitrogen for 3 h. The reaction was considered complete, due to the absence of water produced in the reaction medium. (PGLD-Ch)B membranes of 0.1–0.2 mm thickness were prepared using the solvent casting technique from dimethylacetamide solutions onto Teflon plates at 60°C. Final drying was done in a vacuum oven at 60°C for 24 h. The resulting membranes were washed with physiological solution and finally dried under vacuum. PGLD-ChB membranes with 10 and 30 wt% of PGLD content, PGLD-ChB-10 and PGLD-ChB-30, respectively, were obtained.

Spectroscopic and thermal characterization of (PGLD-Ch)B

The (PGLD-Ch)B dendrimer was characterized by spectroscopic techniques (^{1}H-NMR, ^{13}C-NMR), GPC and DSC. The average molecular weights were characterized by gel permeation chromatography (GPC) using Waters μSTYRAGEL columns of pore sizes 50, 10^2, 10^3 and 10 Å, DMF as the mobile phase and a flow rate of 1.0 ml/min. Poly(propylene oxides) from Aldrich with standard molecular masses of 1, 2, 4, 8 and 12 kDa were used for calibration.

^{1}H-NMR and ^{13}C-NMR spectra were recorded from deuterated methanol solutions (200 g/l) on a Bruker ARX 300 spectrometer.

Differential Scanning calorimetry (DSC) measurements were carried out on a Perkin-Elmer 7 series thermal analysis system in the temperature range -100°C to 20°C at a heating rate of 5°C/min.

Water diffusion on (PGLD-Ch)B membranes

The swelling capacities of the obtained (PGLD-Ch)B membranes were measured by ATR–FT-IR spectroscopy [30]. A thin layer of (PGLD-Ch)B was deposited in a germanium crystal and exposed to water vapor for water diffusion measurements. In the ATR–FT-IR experiment the mass transport properties cannot be measured directly as they are convoluted with the evanescent field. Thus, the convolution of Cranks solution to Fick's second law with Harrick's equations for the evanescent field strength allowed the calculation of the diffusion coefficient.

Biological properties of (PGLD-Ch)B membranes

The cytotoxicity of (PGLD-Ch) B extracts was evaluated against Chinese hamster ovary (CHO) cells, ATCC CHO k1 (American Type Culture Collection, ATCC), according to ISO guidelines [31]. Serially diluted PGLD-ChB extracts were added to a CHO cell culture. CHO cells were grown in Eagle minimum essential medium with fetal calf serum (MEM-FCS) and 1% penicillin-streptomycin solution, in a plastic tissue culture dish at 37°C and atmosphere of 5% CO_2. After monolayer propagation, the culture medium was removed and the cells were washed with calcium and magnesium phosphate saline buffer. The culture was treated

with 0.25% trypsin solution to detach the cells from the culture tissue. After trypsinization, the cells were transferred to a screw-capped plastic tube, centrifuged and washed twice with calcium and magnesium-free phosphate saline buffer. The cells were resuspended in MEM-FCS and adjusted to give 100 cells/ml. A volume of 2 ml of this cell suspension was seeded to each 60-mm-diameter assay culture dish and incubated for about 5 h for adhesion of the cells. The culture medium was then replaced by 5 ml fresh MEM-FCS in the control plates, and by undiluted (100%) and successively diluted extracts (50, 25, 12.5 and 6.25%), in culture dishes with the adhered cells. All concentrations were tested in triplicate. After incubation of the culture dishes for 7 days (37°C, 5% CO_2) for cell colony formation, the cytotoxicity was evaluated quantitatively, based on the cell viability. After the incubation time, the medium was removed from the dishes and after fixation with buffered saline formalin solution, the colonies were stained with Giemsa. The number of visible colonies on each dish was counted and compared with the number of colonies in the CHO control dish. Phenol solution (0.02%) and polyethylene extract (60 cm^2 in 60 ml MEM-FCS) were used as positive and negative controls, respectively. The results were expressed as percent cell survival from control per volume of extract tested.

The testing of bacterial susceptibility was performed using the agar diffusion method and the antimicrobial activities against *Staphylococcus aureus* and *Pseudomonas aeruginosa* of (PGLD-Ch)B were determined. The *S. aureus* (ATCC 25922) culture was grown overnight on Mueller–Hinton agar. Bacteria were selected in this plate using a sterile wooden inoculating stick, and a bacterial suspension of approximately 1×10^8 colony-forming units (CFU)/ml was prepared in 0,1% peptone water. The optical density measurement was used to approximately the bacterial concentration. The bacterial suspension was diluted to 10^7 CFU/ml in tryptic soy broth (TSB). Serial dilutions of the TSB suspension were made and plated on Mueller–Hinton agar in order to determine the concentration of bacteria in the inoculum. A similar procedure was used for the culture of colonies of *P. aeruginosa* (ATCC 27853) initially isolated from denture plaque. The minimum inhibitory concentration (MIC) determination was performed using dilution method in Mueller–Hinton agar with an inoculum of approximately 10^4 CFU/spot, following recommendations of the HCCLS (National Committee for Clinical Standards) for sensitivity testing. The results were read after overnight incubation at 37°C. The ratio of the colony numbers for the media containing the polymer (M) and those without polymer (C) was taken as surviving cell number and by this value the antimicrobial activity was evaluated.

The *in vivo* experiments were carried out using 16 female Wistar rats weighing 200 g. On the back of each rat, four incisions of about 3 cm long each were made through the skin in the underlying muscular tissue layer under aseptic conditions [32, 33]. Chitosan, (PGLD-Ch)B-10 and (PGLD-Ch)B-30 membranes were placed over the incisions, ensuring that the wounds were completely covered. The fourth incision was used as control. In the same way, chitosan, blends of glycerol 10%–

chitosan (Gly-Ch-10) and glycerol 30%–chitosan (Gly-Ch-30) were studied. After different periods of time, i.e., 3, 7, 15 and 30 days, histological evaluations using hematoxylin-eosin staining were performed to assess tissue response to membranes used and progression of healing.

RESULTS AND DISCUSSION

Synthesis of polyglycerol dendrimer

In this work, the polymerization of glycerol to polyglycerol was carried out in alkaline medium to form the dendrimer core. The reaction is the result of the nucleophylic attack of the hydroxyl groups to the glycerol with water elimination, according to the statistical substitution of two equivalent primary hydroxyls and one secondary hydroxyl group. Thus, if the primary hydroxyls are the only ones concerned in the reaction, the products are linear, but if the secondary hydroxyl groups are also involved, branched chains are formed. It was found that the polymerization of glycerol attained a high percentage of the conversion of the glycerol to poly(glycerol). The PGL was characterized by GPC which showed a conversion degree of 70% and a molecular mass of 450 Da was obtained after reaction time of 25 h.

The poly(glycerol) dendrimer with a highly branched structure and a large number of hydroxyl end-groups was synthesized in alkaline medium using a recently prepared polyglycerol core as initiator and glycidol (Fig. 1). The functionality of PGLD was controlled by varying the degree of polymerization, since on average each monomer in the hyperbranched structure contributed one hydroxyl group. Analysis of the PGLD functionality and evolution on their molecular weight by the hydroxyl value (N_{OH}) measurements suggest that no side reaction occurs during the reaction for PGLD synthesis [34].

^{13}C-NMR spectroscopy was used to verify the relation between the architecture and the degree of branching of the PGLD dendrimer. Since all hydroxyl groups on the polyglycerol core remain potentially active in the course of the polymerization, the resulting structure consists of dendritic (D), linear (L) and terminal (T) units that can be incorporated at each position of the structure. In this sense, if the secondary hydroxyl group has propagated, the polymer chain is attached to a glycerol-like unit, and a linear 1,3-unit ($L_{1,3}$) is formed. If the primary hydroxyl group has undergone propagation, the corresponding linear 1,4-unit ($L_{1,4}$) is generated. If both hydroxyl groups have reacted with monomer a dendritic unit (D) is formed and the polymeric chain is bonded to secondary hydroxyl groups. If a monomer unit has been deactivated by proton exchange or by the final addition of acid, a terminal unit (T) with two hydroxyl end groups may be formed.

The ^{13}C-NMR signal assignments are summarized in Table 1. The degree of branching (DB) was calculated on the basis of the intensity of ^{13}C-NMR signals

Table 1.
^{13}C-NMR assignments of the PGLD synthesized in this work.

Region	Structural unity	Chemical shift (ppm)	Relative integral
$L_{1,3}$	CH_2OH	62.9	1.00
	CH_2	71.2	
	CH	81.6	
D	CH	80.2	1.31
	CH_2	73.0	
	CH_2	72.4	
$L_{1,4}$	CH_2	74.0	6.02
	$CHOH$	70.9	
2D, 2T		72.0–73.5	12.45
$L_{1,3}$, $L_{1,4}$		70.5–72.0	3.75
T	CH_2OH	64.5	4.31
	$CHOH$	71.3	
	CH_2	72.4	
$L_{1,3}$		62.0–63.5	1.14

from the fraction of structural units using the previously derived equation [35]:

$$DB = \frac{2D}{2D + L_{1,3} + L_{1,4}}. \tag{1}$$

The value of DB measures the suitability of a hyperbranching reaction to create dendritic structures. The linear polymers not have a degree of branching (DB = 0) while in the perfect dendrimer DB is equal to 1. From the integration of signals of various methyl groups, a DB value of 0.85 was determined.

Synthesis of PGLD-Ch and (PGLD-Ch)B complexes

Chitosan is a polysaccharide derived from chitin, a linear chain of acetylglucosamine units. An important characteristic of chitosan is its capacity to stimulate the polymorphonuclear leukocytes actuating in the would-healing process [36]. The polymorphonuclear leukocytes show an enhanced phagocytic activity upon interaction with chitosan, which in turn causes an increase in the production of bactericidal oxygen species at cellular level. In this sense, the incorporation of chitosan to polyglycerol dendrimers results very attractive to obtain antimicrobial coatings.

It is well known that chitosan has a pK_a value of 6.5, due to its primary amino groups. As a result of that chitosan is soluble in pH values of lower than 6.0 and many of its applications in physiological conditions may not be performed, since they will trigger an immediate precipitation. An approach towards improving the solubility of chitosan at neutral pH is the chemical derivatizion of chitosan *via* carboxymethylation, since chitosan has nucleophilic primary amine and hydroxyl groups at nearly every repeating sugar residue that can be activated. In this work, the hydroxyl group of chitosan was O-carboxymethylated with chloroacetic acid in
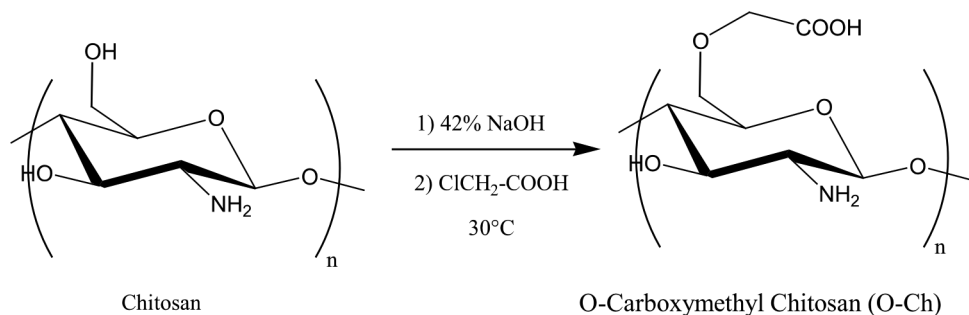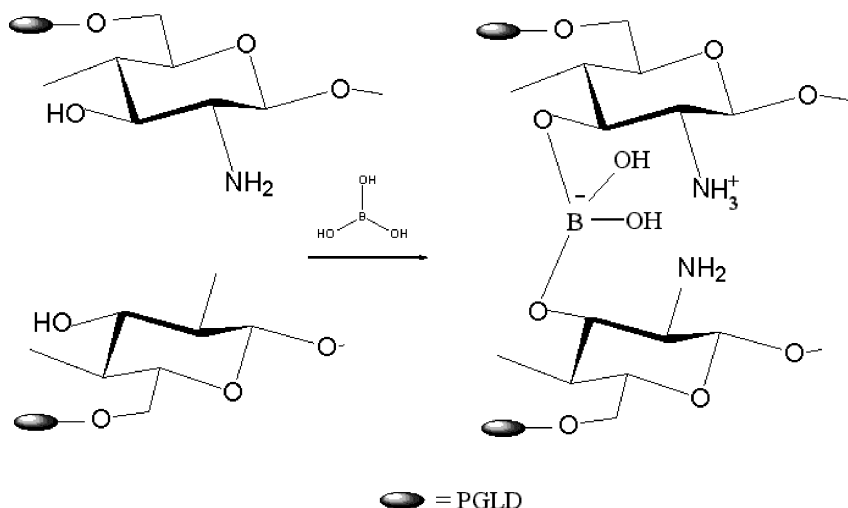
Scheme 1.
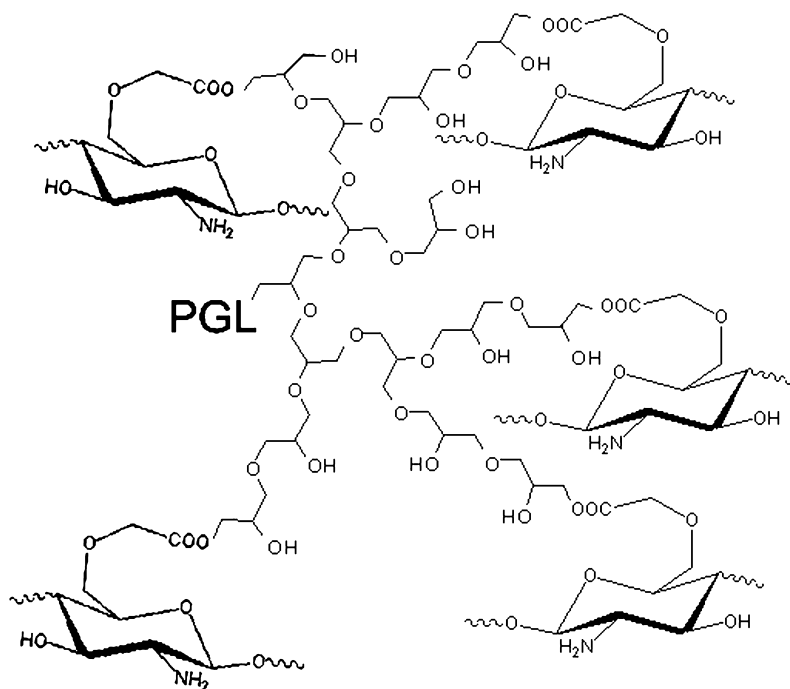
Scheme 2.

aqueous sodium hydroxide [37] (Scheme 1) followed by the coupling of O-Ch to PGLD dendrimer using dimethylsulphate to render PGLD-Ch (Scheme 2).

In this work, a logarithmic relationship between the chitosan substitution degree on PGLD and the calculated theoretical substitution degrees was obtained (results not shown here). The results suggest that the desired levels of substitution may be attainable by varying the mol ratios of chitosan and PGLD, thereby providing means of an external control of the PGLD derivatization reaction. Analyses of the amine groups content on the dendrimer after O-carboxymethylation indicate a value of approximately 58 mmol/g polyglycerol dendrimer for O-Ch on PGLD dendritic structure, equivalent to 60% of carboxymethylation in chitosan.
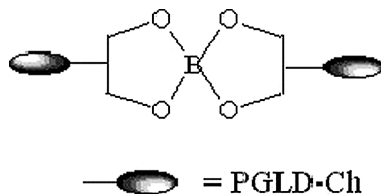
Scheme 3.

Boron complexes have been commonly used in pharmaceutical applications as a mild antiseptic for burns and surface wounds and they are a major ingredient in eye lotions. There are attempts to use boron complexes to modulate the response to antigens of key immune cells controlling the inflammatory process, as well as preventing the calcium loss and bone demineralization in postmenopausal women due to the mimicking effects of the inorganic element of estrogen in post-menopausal women [38–41].

It is well known that polyhydroxylated compounds can react with boric acid in aqueous solutions to form stable borated ester-like complexes, resulting in uncharged trigonal complexes or dissociated tetrahedral complexes corresponding to the sequence of the hydroxyl groups in their molecules [42–45]. In the present work, PGLD-Ch was complexed with boric acid to form esters, (PGLD-Ch)B, in according to the reaction pathway depicted in Scheme 3.

The hydroxyl (C-6) and amine chitosan groups are two sites available for boron binding. The reactivity of the amine groups is convenient as they allow polyglycerol-chitosan boron complexes to be easily generated. The high amine and hydroxyl functionalities of PGLD-Ch could be favorable to the esterification reactions between boric acid and the dendritic macromolecule providing the formation of the boron-complexed dendrimers. At same time, the vicinal diol functions of the PGLD-Ch structure act as chelating agent for boric acid, by forming cyclic boron esters as shown in Scheme 4.

When PGLD-Ch reacted with boric acid there was a marked increase in the bulk viscosity of the (PGLD-Ch)B. The product was characterized by a Brookfield viscosimeter which showed a viscosity of 1600 cps for a boron content on the dendrimer of 25% (m/m). The high viscosity of the (PGLD-Ch)B may evidence physically that boron can initiate a complex formation with PGLD-Ch dendrimer.

Scheme 4.

The glass transition temperature (T_g) was determined by differential scanning calorimetry (DSC). The measurements showed that both PGLD-ChB-10 and PGLD-ChB-30 are flexible polymers with T_g values of -19 and $-26°C$, respectively. The low T_g values observed for these materials suggest that the complexed dendrimer may be used as an interesting coating with antimicrobial properties.

Swelling properties

The water diffusion or permeation in polymers is frequently accomplished by gravimetry measurements, following mass uptake curves during the direct immersion or by establishing a concentration gradient across the membrane and monitoring the permeation transient. However, at high water sorption levels the increase in film thickness produces a shift of the sorption kinetics towards the Fickian model.

FT-IR is an *in situ* spectroscopic technique particularly suitable for high water sorption levels without the shift on the sorption kinetics. The ATR–FT-IR spectrum of (PGLD-Ch)B-30 dendrimer exposed to water at different intervals of time is shown in Fig. 2. The characteristic band at 3400 cm^{-1} was attributed to the water absorbed by the PGLD-Ch dendrimer. In order to determine the maximum swelling degree Q_{max}, the water absorption band was integrated. In Fig. 3, the Q_{max} values characteristic of a superabsorbent hydrogel are shown as a function of time. The (PGLD-Ch)B complexes exhibited high swelling degree without dissolution relatively to the chitosan films or their physical mixtures with boric acid and glycerol. No significant differences were found when (PGLD-Ch)B-10 samples were studied.

Antimicrobial properties

Biological properties of dendrimers are crucial because of the growing interest in using them in biomedical applications. Many potential applications of dendrimers are based on their unparalleled molecular uniformity, multifunctional surface and presence of internal cavities. These specific properties make dendrimers suitable for a variety of high technology uses including biomedical and industrial applications.

Studies of bacterial sensitivity towards the PGLD-ChB dendrimer were carried out and the diameter of the inhibition area of the bacteria using the dendrimer and boric acid was estimated. *S. aureus* was chosen for this work because it is a common and serious pathogen isolated from biomaterials infections. *P. aeruginosa*
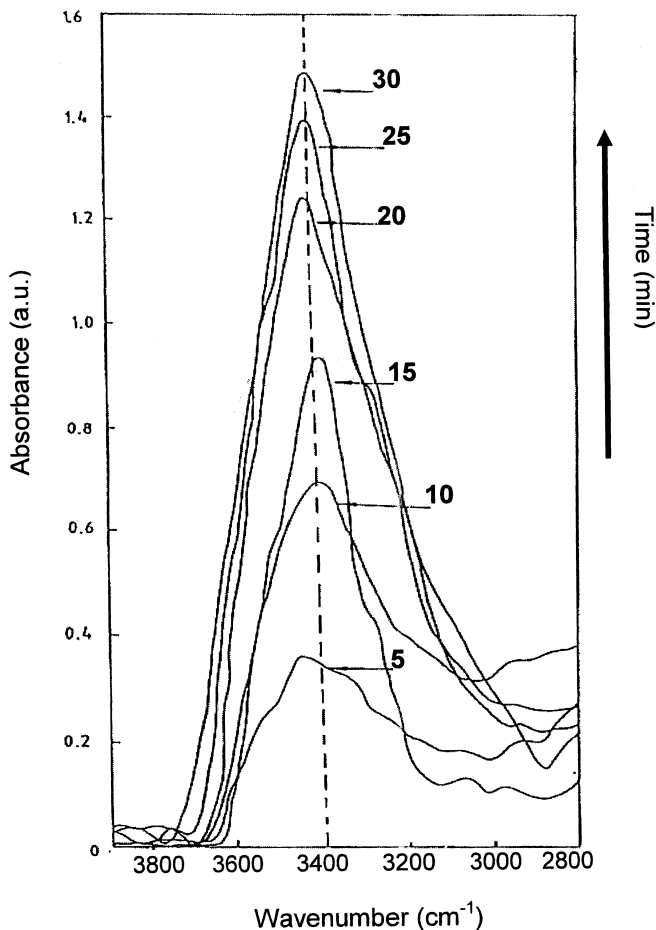
Figure 2. ATR–FT-IR spectra of PGLD-ChB dendrimer exposed to water for different intervals of time.

is the most frequent Gram-negative etiologic agent associated with infections of indwelling catheters and foreign body implants.

The capability of the prepared (PGLD-Ch)B to inhibit the growth of the tested microorganisms on solid media is shown in Table 2. It was found that the diameter of inhibition zone varied according to the to the test bacteria. As can be seen from the presented data in Table 2, the inhibition area of the dendrimer is higher than for the boric acid. The uptake of boron as borate $[B(OH)_4]^-$ anion in the dendrimer to gives a symmetrical borate anion as illustrated in Scheme 3. In this study, the stability of the boron complexes appears to be swelling dependent. Since the boric acid reacts with polyhydroxyl compounds as a Lewis acid, the complex stability may be dependent of the acidity of the chelate ligands. The highly hydrophilic character of PGLD results in a dense gel. The high water uptake may displace the equilibrium constant of the (PGLD-Ch)B complex, in this form accelerating
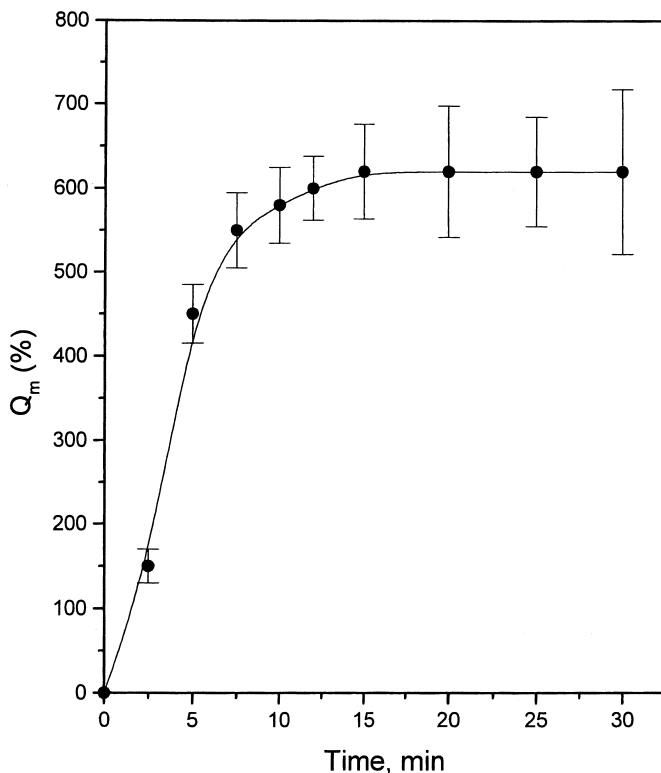
Figure 3. Swelling degree (Q_{max}) of PGLD-ChB dendrimer measured by ATR–FT-IR after integration of the water absorption band.

the boron release process from the polymer matrix. In this sense, the wound exudate containing water, enzymes and inorganic anions may be absorbed by the (PGLD-Ch)B matrix, while at the same time the boron component of the polymer structure is released into the wound in small amounts at a defined rate. We have determined the boron delivery from the (PGLD-Ch)B matrix by using inductively coupled plasma atomic emission spectrometry (ICP-AES) (delivery kinetics is not shown in this work). The *in vitro* (phosphate-buffered saline (PBS), pH 7.4, 37°C) boron-release profiles (not shown) indicated that the amount of boron delivery per gram of (PGLD-Ch)B matrix was 5 μg/g after 600 min. Then, the boron delivery is much slower because of the stability of the complex. The slow release of boron from (PGLD-Ch)B matrix may be used effectively as antimicrobial agent reducing in this form the risk of resistance.

The growth inhibitory effect was quantitatively determined by ratio M/C of the surviving cell number (M) in the medium containing the dendrimer to that without the macromolecule (C). As shown in Fig. 4, the growth inhibitory effect of (PGLD-Ch)B-30 dendrimer differed among the bacterial strains. The inhibitory effect becomes stronger in the order *Staphylococcus aureus* > *Pseudomonas aeruginosa.* No differences were found when (PGLD-Ch)B-10 was studied. The results show

Table 2.
Diameters of inhibition zones produced by 20 mg (PGLD-Ch)B or boric acid against different test bacteria by the plug method after 24 h incubation at 37°C.

Material/Bacteria	Inhibition zone diameter (mm)
(PGLD-Ch)B with *Staphylococcus aureus*	34 ± 2
(PGLD-Ch)B with *Pseudomonas aeruginosa*	25 ± 1
Boric acid with *Staphylococcus aureus*	15 ± 2
Borid acid with *Pseudomonas aeruginosa*	10 ± 1
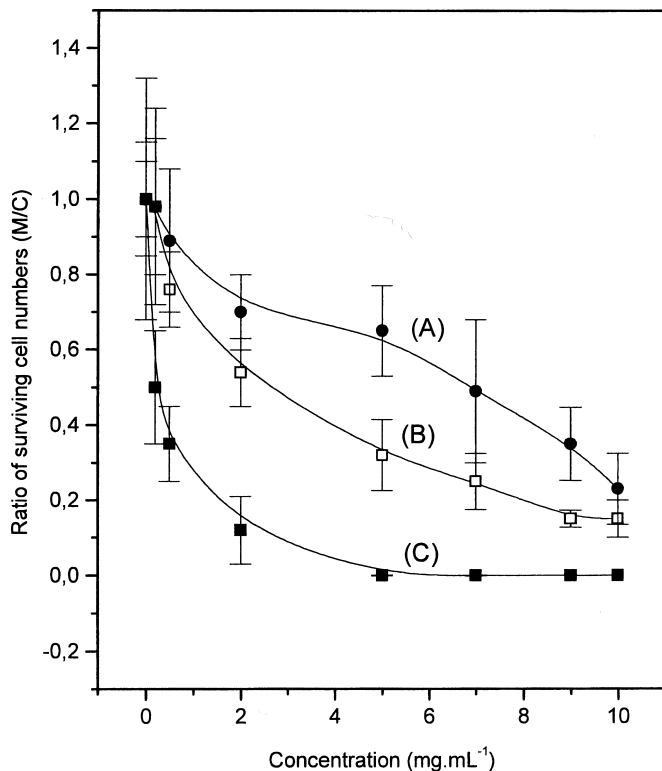
Figure 4. Inhibition of growth as a function of (PGLD-Ch)B-30 concentration. (A) Boric acid, (B) *Pseudomonas aeruginosa* and (C) *Staphylococcus aureus* (C).

also that the inhibition increased by increasing the concentration of the polymer, which may indicate a strong interaction with the cell membrane of the micro-organism. The results shown that two mechanisms may be responsible for the antimicrobial property of (PGLD-Ch)B, boron delivery from the (PGLD-Ch)B and a polycationic nature of the synthesized dendrimer. The cationic nature of (PGLD-Ch)B leads to the interaction with the electronegative bacterial cell surface and the disruption of barrier properties of outer membrane of Gram-negative bacteria might be a second possible mechanism for the antimicrobial action of (PGLD-
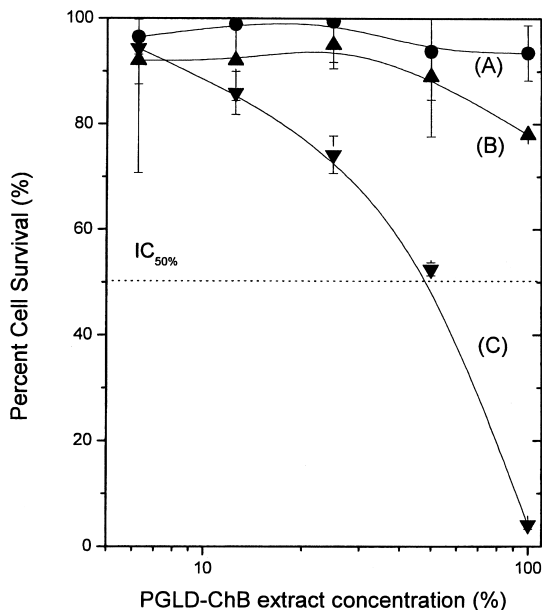
Figure 5. Cytotoxicity of the (PGLD-Ch)B extracts. (A) negative control (UHMWPE), (B) against Chinese hamster ovary cells and (C) positive control (phenol).

Ch)B. However, the exact mode of action of the (PGLD-Ch)B dendrimer on the test microorganism needs more investigation and is actually under study in our laboratories.

Cytotoxicity assay

Under sterile conditions, (PGLD-Ch)B was immersed in the culture medium for 72 h at 37°C without agitation. According to ISO 10993-5 [31], the ratio was determined with the weight of the sample, 0.1 g/ml. Ultra-high-molecular-weight polyethylene (UHMWPE) was used as negative control because it did not exhibit a cytotoxic response. On the other hand, when we tested phenol solution (10 mg/ml) a cytotoxic response was observed and used as positive control. At the end of the incubation period fragments of (PGLD-Ch)B and PE were removed and the so-called extracts were ready to be tested. The (PGLD-Ch)B cytotoxicity level was relatively low (Fig. 5) and supported further studies with this material using animals models, in order to gain insight into the material behavior within the biological media.

In vivo *behavior*

The healing process for each wound treated by membrane application in rats progressed satisfactorily without any apparent complications. No evidences of necrosis were found. At 3 days post-operation, all the wound-treated membranes

displayed no inflammation at all. At 7 days post-operation, an external cicatrisation was observed in all the membranes, remaining opened the internal wound. The macroscopic observations at 30 days post-operation showed a complete healing for all the studied materials.

Through microscopic observation it was noted that all the implants in rats interact to some extent with the tissue environment in which they are placed. The important complications of medical devices are largely based on biomaterial–tissue interactions that include both effects of the implant on the host tissues and effects of the host on the implant. Wound healing consists of the restoration of the continuity of living tissue and is an integrated response of several cell types to injury. In this sense, the platelet aggregation and blood clotting, the formation of fibrin and the inflammatory response play the most important role in the normal healing process. In this work, all membrane-treated wounds showed a mild polymorph inflammatory tissue response containing lymphocytes, neutrophils and monocytes, as observed in the surrounding tissue of the membrane material at 3 days post-operation.

The tissue around Gly-Ch-10 and (PGLD-Ch)B-10 implants elicited a dense fibrosis reaction adjacent to the material (Fig. 6a and 6c). For the Gly-Ch-30 membrane, the implant site containing fragments of material, which was stained red due to the eosinophilic character of chitosan (Fig. 6b). The surrounding tissues also presented a typical inflammatory tissue response with formation of blood vessels as visualized for (PGLD-Ch)B-30 in Fig. 6d.

At 7 days post-operation, the evolution was found to be practically uniform, displaying a complete cicatrisation with a normal disruption of the collagen fibers ordering in the dermis. This fact indicates that the healing process proceeds successfully. At 15 days post-operation, the collagen fibers are almost complete regenerated and only a scarce inflammatory response was observed in Gly-Ch-10 (Fig. 7a). In the case of (PGLD-Ch)B samples the best response was found for membranes with 30% of PGLD content (Fig. 7c) which exhibited higher synthesis of collagen fibers than the sample containing 10% of PGLD content (Fig. 7b). Finally, at 30 days post-operation, the inflammatory cells surrounding the wound area disappeared and wounds were completely healed. No evidence of fragments of membranes was found in all the cases, the degradation being complete. The absence of fragments of membranes may indicate a good biodegradability of the (PGLD-Ch)B and may be favoured by water diffusion and the highly hydrophylic nature of the complex. The last one is of great importance since highly quantities of sorbed water may promote a diffusional mobility of the enzymes presents in the physiological fluid in the polymer matrix. The high enzymes mobility make the sensitive liaisons more accessible to the hydrolytic action of the media.

CONCLUSIONS

In this work, the preparation of a dendrimer containing boron based on chitosan and polyglycerol is achieved. Our results have shown that complexation of PGLD-Ch
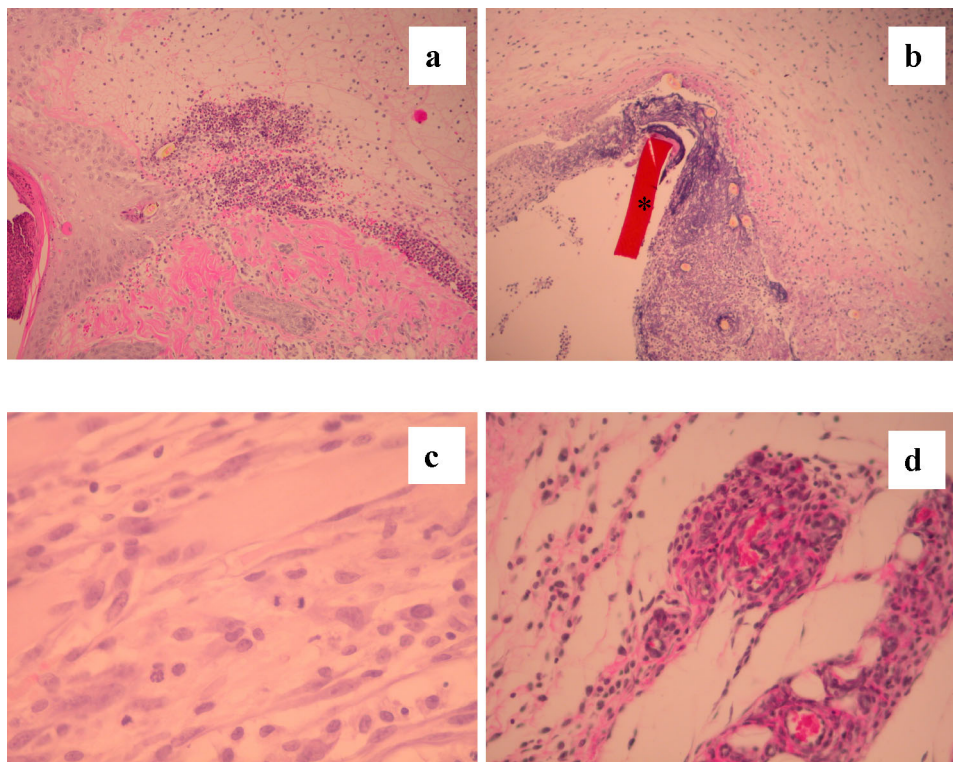
Figure 6. Micrographs stained with hematoxylin-eosin obtained at 3 days postoperatively, wound treated with (a) Gly-Ch-10 membrane (original magnification × 20); (b) Gly-Ch-30 membrane (original magnification × 10), the asterisk (*) indicates the stained membrane; (c) (PGLD-Ch)B-10 membrane (original magnification × 40) and (d) (PGLD-Ch)B-30 membrane (original magnification × 20).
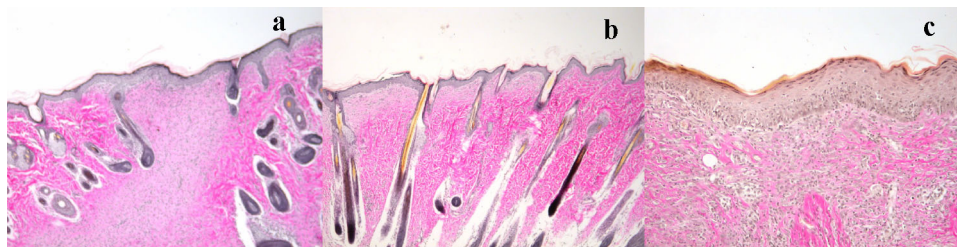

Figure 7. Micrographs stained with trichrome obtained at 15 days postoperatively, wound treated with (a) Gly-Ch-10 membrane (original magnification × 4); (b) (PGLD-Ch)B-10 membrane (original magnification × 10) and (c) (PGLD-Ch)B-30 membrane (original magnification × 4).

with boron provides an interesting way to produce antimicrobial coatings. The synthesized (PGLD-Ch)B exhibited lower cytotoxicity, suggesting that the macromolecule is a biocompatible material. Preliminary studies of the antimicrobial activity suggests that (PGLD-Ch)B may be useful to prevents the bacterial contamination

and may act effectively against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Additionally, the results show that the inhibition increased by increasing the concentration of (PGLD-Ch)B, which may indicate a strong interaction with the cell membrane of the microorganism. The boron delivery or the interaction of the polycationic (PGLD-Ch)B might be other factor responsible for the dendrimer antimicrobial property. The best *in vivo* response was found for (PGLD-Ch)B-30 membranes which exhibited higher synthesis of collagen fibers than (PGLD-Ch)B-10. The biodegradation observed in the *in vivo* assays can be explained by the highly hydrophylic nature of the (PGLD-Ch)B.

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