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SYNTHESIS AND BIOLOGICAL EVALUATION OF BORONATED POLYGLYCEROL DENDRIMERS AS POTENTIAL AGENT FOR NEUTRON CAPTURE THERAPY

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ABSTRACT

In this work, the polyglycerol dendrimer (PGLD) generation 5 was used to obtain a boronated macromolecule for boron neutron capture therapy (BNCT). The PGLD dendrimer was synthesized by the ring opening polymerization of deprotonated glycidol using polyglycerol as core functionality in a step-growth processes denominated divergent synthesis. The PGLD dendritic structure was confirmed by gel permeation chromatography (GPC), nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR) and matrix assisted laser desorption/ionization (MALDI-TOF) techniques. The synthesized dendrimer presented low dispersion in molecular weights (M_w/M_n = 1.05) and a degree of branching of 0.82, which characterize the polymer dendritic structure. Quantitative neutron capture radiography (QNCR) was used to investigate the boron-10 enrichment of the polyglycerol dendrimer. The *in vitro* citotoxicity to Chinese hamster ovary cells of ^{10}B-PGLD dendrimer indicate lower cytotoxicity, suggesting that the macromolecule is a biocompatible material.

1. INTRODUCTION

The successful application of boron neutron capture therapy (BNCT) for cancer requires the preferential concentration of significant quantities of the stable ^{10}B isotope within tumors over normal healthy tissues. The slow evolution of effective drug-targeting methodologies for the selective delivery of sufficient amounts of boron to cancer cells remains as an important obstacle to the development of this therapy [1].

Current research is focused primarily on the identification of superior boron compound biomaterials for the delivery of boron to tumors for application in BNCT. In addition, the developments of new boron-containing polymers have being investigated as a means to increase the quantity and selectivity of boron delivery [2].

In the recent past it has been found that the properties of highly branched macromolecules denominated dendrimers has also a great impact on the biomedical applications. Due to their mimicry of globular proteins and regular micelles, a rapid increase of interest to use dendrimers in medicine has been observed [3].

In the present study we have investigated the synthesis of 5rd generation polyglycerol dendrimers containing a ^{10}B concentrations necessary for the BNCT treatment. The synthesized polyglycerol dendrimers are monodisperse, have nanometric structure and size-controlled diameter with a large number of hydroxyl groups at its periphery.

2. EXPERIMENTAL

2.1. Synthesis of boronated polyglycerol dendrimers

The polyglycerol dendrimer (PGLD) was prepared by following the divergent synthetic methodology due to the control degree 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 polyglycerol core (PGL) was synthesized by glycerol 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 (PGL) 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 weight. HPLC analysis was performed on a JASCO PU-980 Intelligent HPLC pump and 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^{-1} and 30 ºC.

The synthesized PGL core 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. After completion of the reaction, termination was carried out by addition of a drop of acidified methanol. PGLD 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 weight cutoff in the range of 25,000 g.mol^{-1}, Sigma) and subsequently dried for 15 h at 80 ºC in vacuum.

The dendrimer (PGLD) was obtained as a transparent and viscous liquid at room temperature (1,250 cps at 25 ºC). After purification, the nature of the PGLD structure was characterized by GPC (HPLC 510, Waters), ^{13}C-NMR (Bruker 500 MHz) and MALDI-TOF spectroscopy (Bruker, BIFLEX^{TM}).

A boronated dendrimer was prepared by the reaction of PGLD with boric acid (^{10}B) in mole ratio of 2:1. A solution containing 1 mmol of PGLD and 1 mol of boric acid was refluxed in dioxane at 100°C during four hours with the distillation of the dioxane-water azeotrope during two hours. Residual dioxane was removed by heating to 120°C under vacuum (10^{-6} mmHg), leaving boronated PGLD (^{10}B-PGLD) as product.

The boron-10 content in the polyglycerol dendrimer was investigated by quantitative neutron capture therapy (QNCR). The ^{10}B-enriched PGLD samples were introduced into polyethylene bags. After evacuating the bags, the samples were exposed to a thermal neutron flux of 3.6×10^6 nscm^{-2}.s^{-1} over a period of 35 min and Makrofol E as detector.

2.2. Biological ^{10}B-PGLD evaluation

2.2.1. Citotoxicity

The cytotoxicity of ^{10}B-PGLD extracts was evaluated against Chinese hamster ovary (CHO) cells, ATCC CHO k1 (American Type Culture Collection, ATCC), according to ISO guidelines [4]. Serial diluted PGLD-ChB extracts were added to a CHO cell culture. CHO cells were grown in Eagle minimum essential medium – fetal calf serum (MEM–FCS) and 1% penicillin-streptomycin solution, in a plastic tissue culture 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 5mL of 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 the cell colonies 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.

2.2.2. Histocompatibility

Sixteen female Wistar rats weighting 200 g were used in the *in vivo* experiments. On the back of each rat, four incisions about 3 cm long each were made through the skin up to the underlying muscular tissue layer under aseptic conditions. Boronated ^{10}B-PGLD was placed over the incisions, ensuring that the wounds were completely covered. After different period of time, i.e: 3, 7, 15 and 30 days, the rats were sacrificed by using an excess of diethyl ether. Implants with surrounding tissues were carefully dissected from the dorsal muscle site at the corresponding times. Histological evaluations using hematoxylin-eosin staining technique were performed to assess tissue response to membranes used and progression of healing. The samples were immersed-fixed in 10% (v/v) formalin in 0.1 M phosphate buffer (pH = 7.4). In the histopathological examination, the fixed samples were embedded in paraffin, sectioned into slices of 5 μm thickness and then stained with hematoxylin and eosin. The prepared sections were examined using optical microscopy (Nikon, Eclipse E 400).

3. RESULTS AND DISCUSSION

The dendritic structure was confirmed by matrix assisted laser desorption/ionization (MALDI-TOF) techniques and nuclear magnetic resonance (^{1}H-NMR, ^{13}C-NMR). The degree of branching, molecular weights, polydispersity index and hydroxyl number results are presented in Table I. The low dispersion in molecular weight (M_w/M_n = 1.05) and a degree of branching of 0.82 characterizes the PGLD dendritic structure.

Table 1. Physicochemical characteristics of the polyglycerol dendrimer synthesized in this work.

Degree of branching[a]	M_n[b]	M_w[b]	M_w[c]	PI[b] M_w/M_n	OH value (mgKOH.g^{-1})
0.82	16,724	17,564	17,280	1.05	495

[a]Calculated from NMR analysis, [b]Calculated from GPC analysis, [c]Calculated from MALDI-TOF spectroscopy.

It is well known that GPC calibrated with linear standards, underestimates the average numeric molecular weight (M_n) for branched systems. In an effort to be more certain about the polyglycerol dendrimer molecular weights, MALDI-TOF mass spectroscopy was attempted. Satisfyingly, the spectrum showed a Gaussian distribution of peaks centered around 17,280 g.mol^{-1} in a reasonable concordance with GPC analysis.

In order to monitor the mechanism of termination, the hydroxyl values of products were measured using chemical analysis. The value of 495 mg KOH.g^{-1} (Table 1) suggests that in the polymerization system no side reaction occurs during the termination process. Figure 1 illustrates the reaction scheme for the PGLD synthesis.

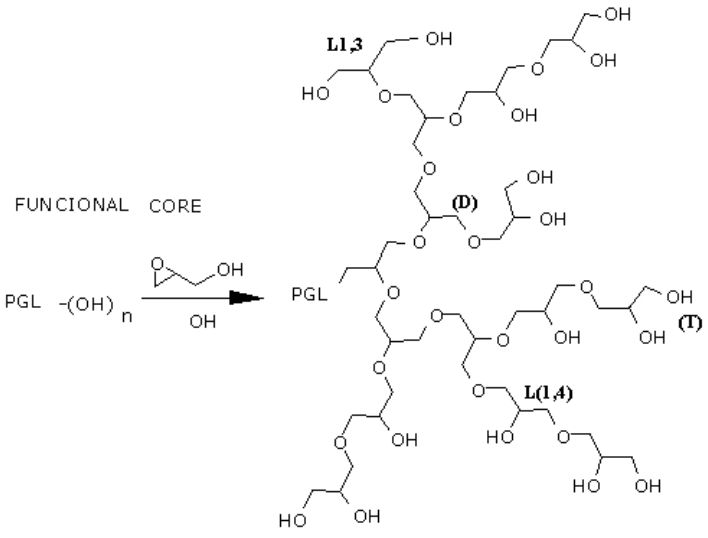

Figure 1. Schematic architecture of the polyglycerol dendrimer (PGLD) synthesized in this work. Examples of terminal (T), linear 1,3 ($L_{1,3}$), linear 1,4 ($L_{1,4}$) and dendritic (D) units are indicated.

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.

The high hydroxyl functionalities of PGLD could be favorable to the esterification reactions between boric acid and the dendritic macromolecule providing the formation of cyclic boron esters as shown by Figure 2.

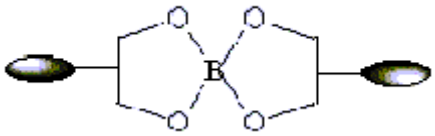

Figure 2. Molecular structure of boronated PGLD dendrimer. B = ^{10}B and = PGLD dendrimer.

Figure 3 shows the *in vitro* citotoxicity assay for ^{10}B-PGLD according to ISO 10993-5 assay. Ultra-high molecular weight polyethylene (UHMWPE) was used as negative control because did not exhibit a cytotoxic response. Phenol solution at concentration of 10 mg.mL^{-1} was used as positive control for the cytotoxic response. The boronated PGLD cytotoxicity level was relatively low (Figure 3) and support further studies with this material using animal's models, in order to gain insight into the material behavior within the biological media.

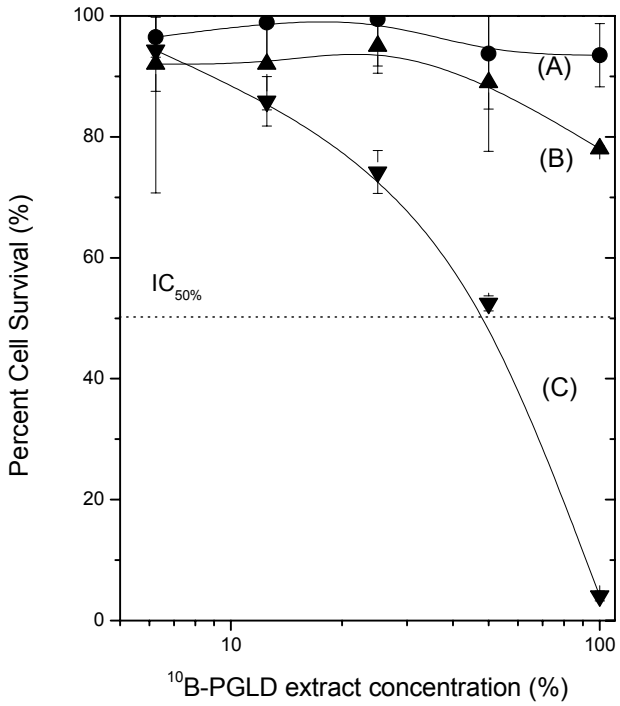

Figure 3. ^{10}B-PGLD extracts cytotoxicity of the (B) against chinese hamster ovary cells. (A) negative control (UHMWPE) and (C) positive control (phenol). Boron-10 content in PGLD: 25 μg/g.

The ^{10}B distribution in PGLD dendrimer was studied by boron neutron capture radiography (BNCR). The BNCR showed highest ^{10}B concentration in function of dendrimer generation (Table 2), suggesting that boronated PGLDB might be a promising biomaterial for use in treatment of neoplasic cells by BNCT. Additionally, the bulk viscosity was measured as a function of various boron concentrations (Table 2). When PGLD reacted with boric acid there was a marked increase in the bulk viscosity of the polyglycerol dendrimer evidencing physically the enrichment of PGLD with boron-10.

Table 2. Mean boron-10 concentration in PGLD dendrimer from different generations.

Dendrimer generation	1	2	3	4	5
^{10}B content (μg/g)	3	11	25	140	180
Viscosity (cps)	263	575	1,250	1570	1,870

In the case of *in vivo* assays, the implanted ^{10}B-PGLD showed higher synthesis of collagen fibers (micrographs are not shown here) without inflammatory cells responses as well as with healing of the wound area.

3. CONCLUSIONS

The results obtained in this work demonstrate that enriched boron-10 polyglycerol dendrimers may be in principle a reasonable route for manufacturing cost effective biocompatible macromolecule for boron neutron capture therapy. The enriched boron-10 dendrimer exhibited lower cytotoxicity to mammiferous cells suggesting that the macromolecule is a biocompatible material.

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