Influence of Crosslinking Agent - Chain Extender Ratio on the Properties of Hyperbranched Polyurethane Structures used as Dendritic Drug Carrier

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Polyurethanes (PU) are versatile chemical compounds with vastly differing biological and mechanical properties. The slight possibility of converting their properties allow them to be used in many applications, particularly for the biomedical and pharmaceutical fields. In this study, lysine diisocyanate ester (LDI), tris(hydroxymethyl)aminomethane (THAM), 1,4-butanediol (BD) and polyethylene glycol (PEG) were used to obtain hyperbranched PU structures. There were studied the size and stability of the PU structures and the thermal behavior of samples. In vitro assays on cells culture were used to observe the cytotoxic potential of obtained products. The sizes and stability of the obtained PU structures and the absence of any cytotoxic potential recommend their usage as drug delivery system.

Keywords: polyurethane, drug-delivery system, cytotoxic potential, zeta potential

Nanoscale materials, with sizes between 1-100 nm, have been developed in the last 55 years, since R. Feynman had the idea to describe a process by observing the ability to manipulate individual atoms and molecules [11]. There are many scientists which consider that the beginning of nanotechnology was in the early twenty century and it can be associated with the discoveries from interface and colloidal science [7, 15]. Different nano- and micro-scale drug delivery systems are often used in the last decade because they can provide therapeutic concentrations of active biological substances at the desired sites while sparing normal tissues, thereby reducing systemic toxicity and enhancing therapeutic efficacy [19].

Prof. Eniola-Adefeso and her team noticed that once nanostructures reach the blood stream, it is very difficult to find an exit; another bad aspect is that red blood cells tend to push large microstructures with diameters of two microns or more towards the blood vessel wall. Inside blood vessels in which blood flows uniformly, as is the case in the arterioles and venules, or pulsed, as happens in arteries, large microstructures reach the wall of the vessel and attached to it. While microstructures are too large to serve as drug carriers on their own, the research team suggested that these could carry nanostructures through the vessel wall and freeing them as an attachment, but the simplest approach would be nanoparticles of different shapes, which can get rid of red blood cells on its own [4].

Polyurethane nanoscale materials may be used as drug delivery systems due to their biocompatibility, hemocompatibility [12, 16], and due to the ease with which can be modified their physico-chemical properties [13]. Polyurethanes are polymer materials obtained in a polyaddition process between an organic phase based on an isocyanate component and an aqueous phase based on a mixture of diols and polyols. The main isocyanates used in the industrial field are aromatics (diphenyl-methane-diisocyanate, toluene-diisocyanate) due to their improved mechanical characteristics while in medical appliances, implants, and drug carriers' domain the scientists avoid the usage of aromatics isocyanates due to their carcinogenic potential. The polyol component is a mixture of diols or diamines with low molecular weights used as chain extenders (ethylene glycol, butanediol, hexanediol, ethanolamine, ethylenediamine etc.), three functionalized compounds used as crosslinking agents (glycerin), and polyether or polyester polyols.

The main aims of this research were to obtain hyperbranched polyurethane structures and to study the influence of THAM/BD ratio upon the physico-chemical properties of these PU structures which can be used as a drug delivery system.

Experimental part

Materials and Methods

Lysine diisocyanate ester (LDI) was purchased from Hangzhou Imaginechem Co. (China Rep.). 1,4-Butanediol (BD) was obtained from Carl Roth GmbH (Germany); polyethylene glycol with M=200 (PEG), tris(hydroxymethyl) aminomethane (THAM), anhydrous tetrahydrofuran (THF), dimethyl-sulfoxide (DMSO), ethanol and acetone were obtained from Sigma Aldrich (Germany). All commercial chemicals were used without purification.

The aqueous phase was prepared by dissolving different ratios of THAM/BD in THF then was added PEG and the mixture was stirred at 400 rpm and maintained at 65°C for 30 min. The organic phase was formed from LDI dissolved in THF (1:20, v/v) and kept in similar conditions before the synthesis.

The organic phase was rapidly poured in the aqueous phase under ultrasound treatment. A powerful ultrasonic

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Sample code	Organic phase, ml		Aqueous phase, ml			
	LDI	THF	THAM	BD	PEG	THF
S01	2.0	40.0	0.4	0.1	2.0	20.0
S02	2.0	40.0	0.3	0.2	2.0	20.0
S03	2.0	40.0	0.2	0.3	2.0	20.0
S04	2.0	40.0	0.1	0.4	2.0	20.0

Table 1
RATIOS OF RAW MATERIALS FOR THE SYNTHESIZED SAMPLES

lab homogenizer UP200S (Hielscher, Germany) was used with the following settings: complete cycles, an average amplitude of the oscillatory system (50%), and the S3 (micro tip 3) standard sonotrode. The temperature was then increased slowly up to 110 °C, and the reaction was continued for 3 h under the same conditions after a procedure already described in literature [3, 21]. After the completion of reaction, the final product solution was dried 12 hours in a hot air oven (PolEko, Poland) at 50 °C to obtain the PU structures as dried powders.

The procedure previously described was used four times in order to obtain samples with different THAM/BD ratios. Table 1 describes the samples obtained and studied in this research.

A very important aspect of this chemical synthesis is the absence of reaction catalyst. PU chemistry often uses amine compounds (tertiary amines: triethylenediamine, dimethylcyclohexylamine, dimethylethanolamine) and/or metal complexes (oragno-metallic compounds based on tin, bismuth, mercury, lead, and zinc) as catalysts [20, 22]. The reaction catalysts assures very short times for PU foams manufacturing (around a few tens of seconds), but in this study the team preferred not to add additional chemicals even if the reaction time was prolonged.

The obtained powders containing PU structures were solubilized in distilled water, ethanol, and respectively in acetone in order to evaluate their solubility and the *p*H values of aqueous solutions. Table 2 presents the solubility of samples determined at 25°C. The *p*H values were evaluated in quadruplicate using an automated TitroLine alpha plus compact titrator (SI Analytics, Germany) and samples aqueous solutions with the same concentration (1.1 g/L) at the same temperature (25°C).

Alcoholic solutions (1.2 g/L) were prepared to evaluate the size and the stability of PU structures. The measurements were done in duplicate using a Cordouan Zetasizer equipment (Cordouan Technol., France) containing a Vasco Particle Size Analyzer and a Wallis Zetapotential Analyzer. There were set the following Vasco Particle Size Analyzer parameters: temperature (25°C), time interval (between 13-22 μs), and number of channels (380-440), laser power (between 40-55%), acquisition mode (continuous), and analysis mode (Pade-Laplace). The following Wallis Zetapotential Analyzer parameters were chosen: cuvette type (plastic, with a wavelength from 380 to 780 nm, visible spectrum), temperature (25°C), resolution (medium, 0.8 Hz), and Henry function (Smoluchowski).

The thermal analysis of samples was performed using a Mettler-Toledo DSC1 instrument (Mettler-Toledo, Switzerland) and aluminium crucibles with pierced caps (samples' weights between 4.11-4.42 mg) in an oxidative atmosphere between 30-300°C with a 5 degree/min heating speed.

There were used human dermal fibroblasts (HDFa, Invitrogen, USA) for the *in vitro* study on cells culture; the fibroblasts were cultured in DMEM medium with 1/5 fetal calf serum (FCS, PromoCell, Germany) and 1/100 penicillin-streptomycin (Pen/Strep, 10,000 IU/mL; PromoCell, Germany). The cells were kept in an atmosphere of 5/100 CO_2 at 37±0.4 °C.

During the viability assay, the fibroblasts were seeded onto a 96-well microplate (5000 cells/plate) and attached to the bottom of the well overnight. After 24 h, 150μL of new DMEM medium with 1/5 FCS, and 1/100 Pen/Strep mixture and the tested substances were added. After an incubation of 24 and 48 hours, respectively, 15 μL of Alamar Blue solution was added and the cells were incubated for 4 h at 37±0.4 °C. Finally, the samples were analyzed with a spectrophotometer at 570 and 600 nm respectively; wells with untreated cells were used as controls. DMSO was used to prepare stock solutions of PU structures samples, and there was not observed any significant effect on cell proliferation even at the highest DMSO concentration (1/1000).

Cells viability was calculated using the following formula [17]:

$$\frac{\varepsilon ox2\, A1 - \epsilon ox1\, A_2}{\varepsilon red1\, A2 - \epsilon red2\, A_1} \cdot 10_0$$

where:
$\varepsilon ox1$ = molar extinction coefficient for oxidized Alamar Blue at 570 nm
$\varepsilon ox2$ = molar extinction coefficient for oxidized Alamar Blue at 600 nm
$\varepsilon red1$ = molar extinction coefficient for reduced Alamar Blue at 570 nm
$\varepsilon red2$ = molar extinction coefficient for reduced Alamar Blue at 600 nm
A1 = absorbance of tested cells at 570 nm
A2 = absorbance of tested cells at 600 nm

Results and discussions

Drug-delivery systems are generally used to improve the delivery and the effectiveness of active biological substances or to prolong the *in vivo* drug activity [5]. In the literature, there are mentioned other roles of drug-delivery systems such as: improvement of the drug efficacy by changing its solubility [6], protection of the loaded substances against external agents (strong acid medium, heat, oxygen exposure, UV radiation, etc.) [2], and targeting of the specific site.

Colloidal suspensions contain submicron particles which usually present a complex distribution of particle sizes; this distribution can be narrow (homogeneous systems), but it is often wide (polydisperse) or the suspension can contain a few different populations with varied sizes (multimodal systems) [1]. Diluted alcoholic solutions of PU structures were evaluated using a Vasco

Sample code	Solubility, g/L			pH value
	water	ethanol	acetone	
S01	1.2	1.5	1.4	6.88±0.04
S02	1.1	1.3	1.6	6.91±0.07
S03	1.1	1.2	1.5	6.92±0.08
S04	1.3	1.4	1.5	6.83±0.05

Table 2
pH AND THE SOLUBILITY OF THE SYNTHESIZED SAMPLES

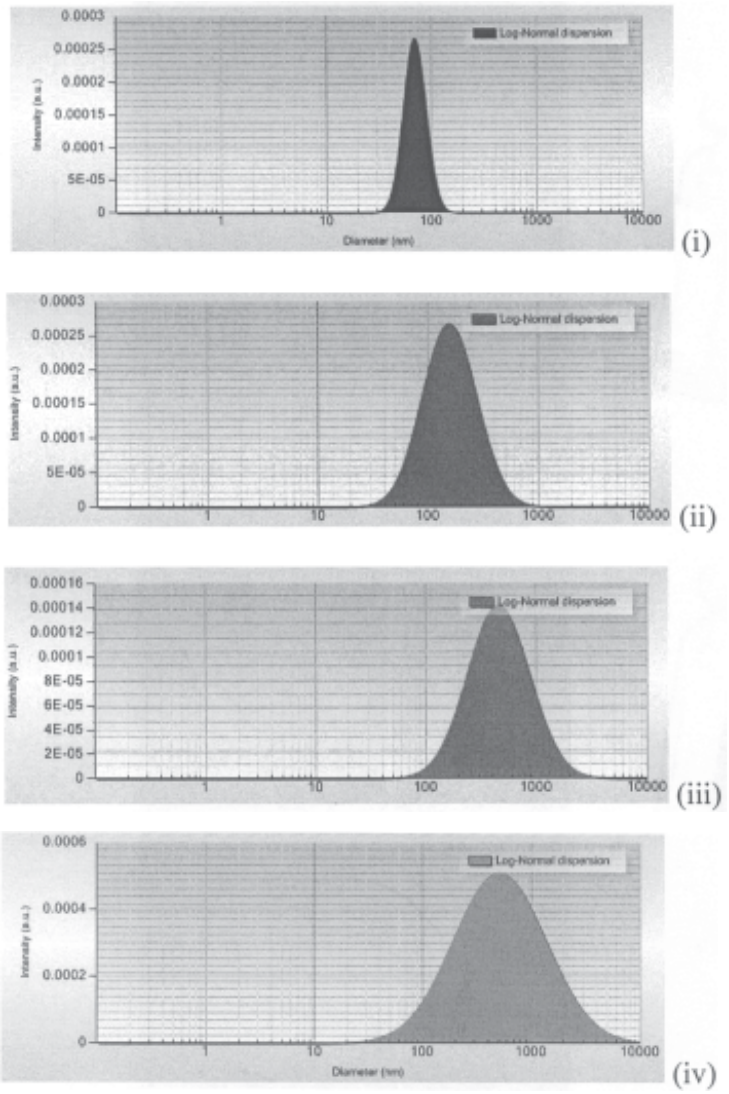

Fig. 1. Size distribution of sample: (i) S01, (ii) S02, (iii) S03 and (iv) S04

Particle Size Analyzer in order to examine their size distribution (fig. 1). The evaluation process is based on the Dynamic Light Scattering or Photon Correlation Spectroscopy, which is considered a powerful and recognized technique used to characterize the size/ hydrodynamic radius of nano-particles dispersed in liquids (colloids) [10].

Polyurethanes with an average size between 10-200μm are essentially used for nano- and micro-particles preparation [8, 14]. The following average values were obtained in this study: 70.47 nm (sample S01), 182.89 nm (sample S02), 558.43 nm (sample S03), and 808.78 nm (sample S04). It is easy to observe that the sizes of PU structures increase with the increasing of THAM/BD ratio (fig. 2). Only the samples S01 and S02 meet the size criteria imposed by literature. It seems that in this experiment, a high THAM/BD ratio lead to smaller particles because when the crosslinking agent amount increase, the packing degree increase too.

The recorded Zeta potential values were situated inside the range of stable particles [9, 18]: 22.6±2.9 mV (sample S01), 27.9±2.1 mV (sample S02), 29.1±1.9 mV (sample S03), and 28.2±1.6 mV (sample S04).

On the DSC diagram of samples (fig. 3) there were no any exo- or endo-thermic peaks. No melting or crystallization process was recorded in this temperature range. The evolution of curves correspond to the hard segment vitrification and devitrification processes. The system vitrified at similar temperature and not depending on the THAM/BD ratio; however there were observed differences on the curve of S01, where some DSC artifacts were obtained too. There was necessary to start the evaluation from negative temperatures to obtain a complete thermal behaviour of samples.

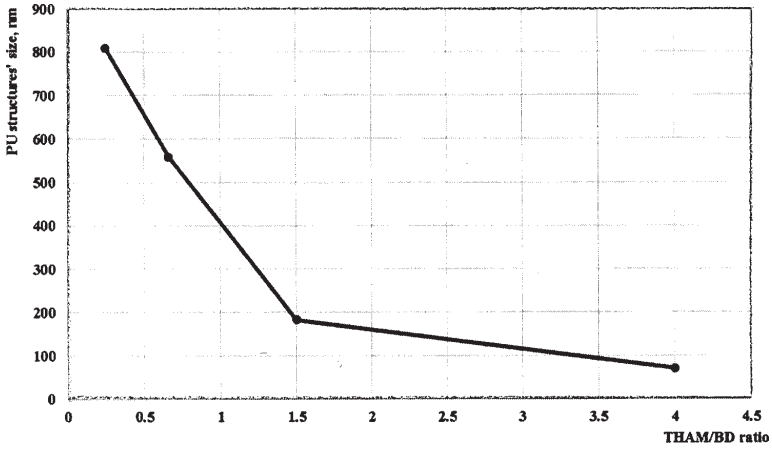

Fig. 2. The influence of THAM/BD ratio on the PU structures size

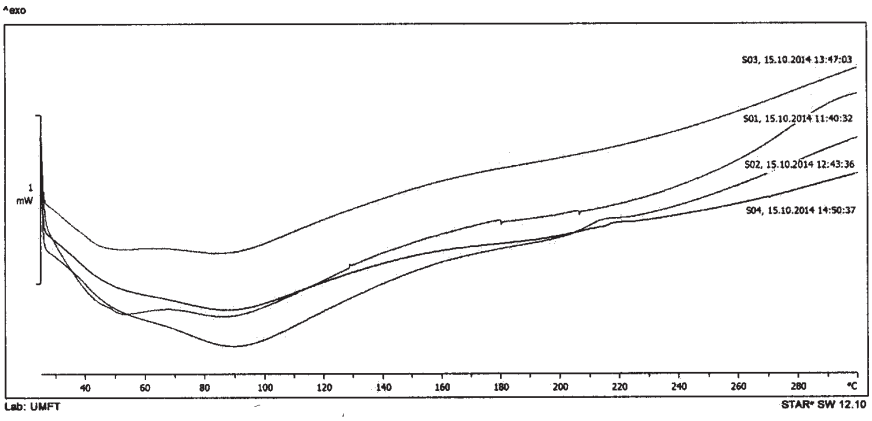

Fig. 3. DSC curves of samples

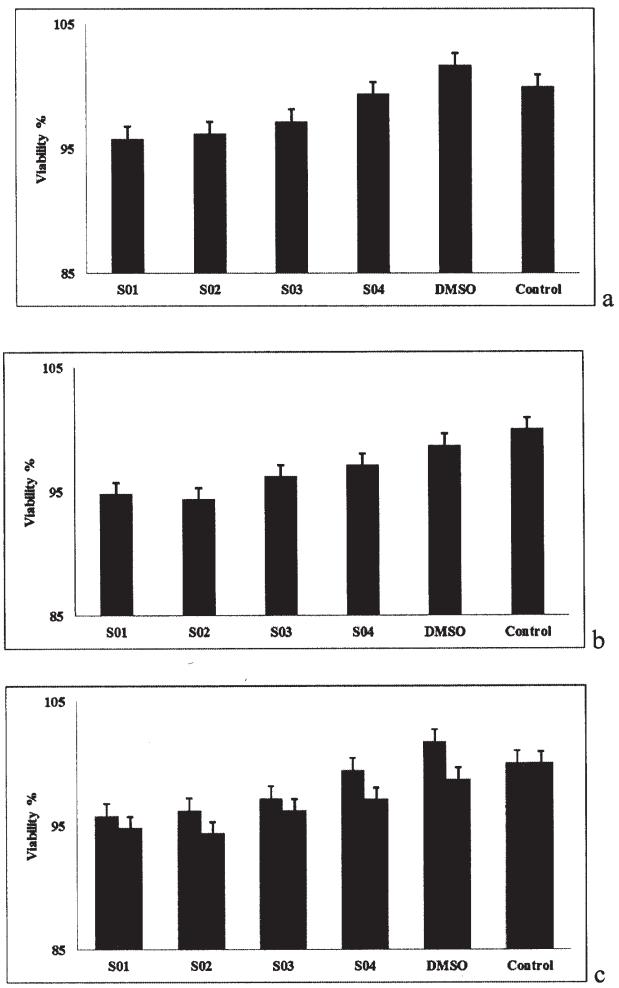

Fig. 4. HDFa viability after (a) 24 h, (b) 48 h, (c) 24 and 48 h exposure on the tested compounds

The PU structures samples were tested on human dermal fibroblasts (HDFa) in order to evaluate their cytotoxic activity. Figure 4 presents the viability of HDFa after exposure to the tested PU structures for 24 h (a) and for 48 h (b). It can be observed that the tested compounds had no major toxic activity on human dermal fibroblast cells neither after 24 h, nor after 48 h.

As figure 4 shows, none of the tested substances had a major proapoptotic effect on HDFa cells. Sample S04 was observed to induce the lowest apoptosis to HDFa cells, only 0.67% of cells being early apoptotic and 2.94% being late apoptotic. The maximum percent of early apoptotic cells was 4.19 % for S01, while the maximum of the late apoptotic cells was 5.61 % for sample S02. The assay shows that none of the PU structures present toxicity on normal human dermal fibroblast HDFa cells. These results can lead to the conclusion that all the formulations obtained could be safe for administration.

Conclusions

Hyperbranched polyurethane (PU) structures were obtained in a polyaddition process between lysine diisocyanate ester and a mixture of butanediol (BD), tris(hydroxymethyl) aminomethane (THAM) and polyethylene glycol. The final products present suitable solubility, *p*H and stability for substances intended to be used as a drug delivery system. There was found that increasing of THAM/BD ratio leads to smaller PU structures and particles below 200 nm can be obtain using THAM/BD ratios higher than 1.5. Cells viability presents lower decreases but the values are not important, so these polymer compounds are safe for administration.

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