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Preparation and characterization of anionic pullulan thermoassociative nanoparticles for drug delivery

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ABSTRACT

Thermoassociative nanoparticles were obtained through the crosslinking reaction of periodate oxidized carboxymethyl pullulan with two difunctional Jeffamines: ED-600 and ED-2003. The nanoparticles were characterized through ^{1}H NMR spectra; their particle size, determined by dynamic light scattering (DLS) presented a bimodal distribution, with dimensions varying as a function of amount and type of crosslinking agent used; transmission electron microscopy photos confirmed the spherical shape of the nanoparticles and their dimensions determined by DLS. Their amphiphilic character was evidenced by retention of the dyes: hydrophobic (Rose Bengal), amphiphilic (Brilliant Blue) and hydrophilic (Vitamin B12). The thermosensitive properties, more pronounced for ED-2003 crosslinked nanoparticles, were evidenced through absorbance variation, fluorescence and DLS measurements as a temperature function. The nanoparticles retain important amounts of anionic (diclofenac: 40–80 mg/g), cationic (methylene blue: 70–125 mg/g) and hydrophobic (alpha-tocopherol: 220–350 mg/g) drugs. The *in vitro* characterization of the drug–polymer conjugates recommends the synthesized nanoparticles as supports for drug delivery.

1. Introduction

Hydrogels, which are composed of water soluble/swellable polymer chains, can be obtained as micro or nanoparticles. Biopolymer-based microgels/nanogels have recently received a great interest in tissue engineering, drug delivery applications, biomedical engineering and biomaterials science, because of their tunable chemical and physical structure, good mechanical properties, high water content and biocompatibility. Nanoparticles are known as submicron-sized, colloidal particles, and their size can be varied from 10 to 1000 nm depending on the end use (Nagarwal, Kant, Singh, Maiti, & Pandit, 2009; Rao & Geckeler, 2011). Nano-sized delivery systems offer many advantages over microparticles. They have higher intracellular uptake, can cross the blood–brain barrier, can be administred into systemic circulation without blocking fine capilaries, they are also able to be targeted to desired organs or tissues by passive or active targeting. These nanoparticles' properties are very useful in cancer therapy, where the size of the delivery system is essentially to target cancer cells through the enhanced permeability and retention effect (EPR) (Maeda, Wu, Sawa, Matsumura, & Hori, 2000). The nanoparticles can be pH- or temperature-sensitive and can release the biomolecules in a controlled manner. Also, they can improve the bioavailability of drugs and thus reduce their side effects.

Among the methods used to develop micro/nanogels one can mention water-in-oil heterogeneous emulsion, aqueous homogeneous gelation, spray drying, chemical or ionic crosslinking, heterogeneous free radical polymerization, controlled/living radical polymerization (Oh, Drumright, Siegwart, & Matyjaszewski, 2008). Many of these methods have been applied for the preparation of biodegradable and biocompatible polysaccharide bases micro/nanogels (Liu, Jiao, Wang, Zhou, & Zhang, 2008). Thus, hyaluronic acid with different molecular weight was crosslinked with a diamine, in the presence of carbodiimide. In aqueous media, the crosslinked hyaluronan nanoparticles may form stable colloid systems (Bodnár et al., 2009; Maroda et al., 2011). Chitosan nanoparticles were prepared by chitosan covalent crosslinking with poly(ethylene glycol)bis(carboxymethyl)ether (Bodnar, Hartmann, & Borbely, 2006) or natural di- or tricarboxylic acids (Bodnar, Hartmann, & Borbely, 2005), as well as by

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ionic crosslinking of poly(ethylene glycol)-grafted chitosan with tripolyphosphate and poly(glutamic acid) (Papadimitriou, Achilias, & Bikiaris, 2012). Microparticles from carboxymethyl pullulan crosslinked with Jeffamines, which are biocompatible difunctional compounds (Marie, Landfester, & Antonietti, 2002), were synthesized by Mocanu, Mihai, Dulong, Picton, and Le Cerf (2012). Degradable hydrogels based on oxidized dextran crosslinked with adipic acid dihydrazide were also reported (Maia, Ferreira, Carvalho, Ramos, & Gil, 2005). A temperature-sensitive amphiphilic polyelectrolyte based on (succinylated pullulan-g-oligo (L-lactide) was synthesized and its ability to form a temperature-modulated noncovalent complex with etanercept (protein drug for rheumatoid arthritis) was studied (Jung, Park, & Na, 2013).

The present paper discusses the synthesis of carboxymethyl pullulan (CMP) nanoparticles by crosslinking of oxidized CMP (containing reactive aldehyde groups) with two difunctional Jeffamines: ED-600 and ED-2003 in aqueous solution, followed by hydrogenation with sodium borohydride. Transparent or mildly opalescent stable colloid systems were obtained in aqueous media at room temperature. To the best of our knowledge, this procedure leading to polysaccharide nanoparticles has not been reported yet. Due to the presence of Jeffamines, which are polyoxyalkyleneamines (polyethylene oxide [PEO], polypropylene oxide [PPO]) with thermoassociative properties, (Belbekhouche, Ali, Dulong, Picton, & Le Cerf, 2011; Belbekhouche, Desbrieres, Hamaide, Le Cerf, & Picton, 2013; Mocanu, Souguir, Picton, & Le Cerf, 2012), the obtained polysaccharide nanoparticles possess both amphiphilic and thermosensitive characteristics, as demonstrated by UV, fluorescence and DLS measurements. The obtained nanoparticles were tested as potential supports for drug release systems.

2. Experimental

2.1. Materials

Carboxymethyl pullulan (CMP) was synthesized in laboratory as previously reported (Mocanu, Mihai, Picton, Le Cerf, & Muller, 2002), and had a degree of substitution with carboxymethyl groups: 0.72. Jeffamines (Jeff) ED-600 and ED-2003 (Aldrich), Rose Bengal sodium salt (RB), Vitamin B12 (B12), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and Diclofenac (DCF) were from Sigma Aldrich, and Brilliant Blue (BB), alpha-tocopherol (α-TF) and methylene blue (MB) were supplied by Fluka.

2.2. Methods

2.2.1. Synthesis of nanoparticles

Oxidized carboxymethyl pullulan was obtained according to a procedure described by Bruneel and Schacht (1993). Briefly, KIO_4 (0.2 g) was added to an aqueous solution of CMP (2 g/50 mL), and the mixture was stirred at room temperature for 24 h in the dark. The unreacted periodate was inactivated with ethylene glycol and the reaction product was dialyzed against water under conductimetric control. The purified solution was lyophilized and the oxidation degree was estimated by reaction with hydroxyl amine hydrochloride followed by potentiometric titration with NaOH (Martwiset, Koh, & Chen, 2006; Scomparin, Salmaso, Bersani, Satchi-Fainaro, & Caliceti, 2011). The values were confirmed by the nitrogen content of the polymers (determined by EDX – Energy Dispersive X-ray method), after the reaction of the aldehyde groups with hydroxyl amine, purification by dialysis, and lyophilization (Kim, Kuga, Wada, Okano, & Kondo, 2000).

The molecular weight of oxidized CMP was determined by gel permeation chromatography. Before GPC analysis, oxidized CMP was reduced with $NaBH_4$, in order to transform the aldehyde groups into hydroxylic ones and obtain a hydrophilic polymer, without substantial changes in the molecular weight of the oxidized polysaccharide. The weight-average (M_w) and number-average molar masses (M_n) of CMP and of its oxidized derivatives were measured with a Shimadzu HPLC System with refractive index detector, and a 10 mm × 250 mm column packed with a 5 μm mixed bed linear Jordi DVB Glucose gel (Grace Davison Discovery ScienceTM). A 100 μL polymer solution (5 mg/mL) was injected to the column and elution was performed with aqueous 1 N NaOH (0.7 mL/min) at 25 °C. Dextran standards were used for calibration.

Synthesis of oxidized CMP-Jeffamine nanoparticles was performed as follows: 0.3 g oxidized CMP was dissolved in 300 mL solution (1 mg/mL) with pH 10, then the desired amount of Jeff was added and the reaction was continued for 24 h at room temperature.

Excess of sodium borohydride (4 moles/mole Jeff) was added to reduce the less stable imine bonds to amine groups, and the reaction mixture was dialyzed against water. The yield of the product recovered after lyophilization was 50–75%.

2.2.2. Characterization of nanoparticles

^1H NMR spectra were recorded on a NMR Bruker Avance DRX 400 spectrophotometer in D_2O.

Transmission electron microscopy (TEM). The size and morphology of the dried oxidized CMP-Jeff nanoparticles were evidenced with a HITACHI T7700 transmission electron microscope operated at 120 kV in high resolution mode. Samples with a concentration of 0.2 mg/mL were dropped onto a carbon-coated copper grid, letting the polymer be absorbed for 3 min, followed by filter paper blotting to remove the excess solution. Staining was performed by exposing the samples to 2 wt% aqueous phosphotungstic acid (PTA) for 30 s, followed by filter paper blotting. The samples were dried overnight under ambient conditions and imaged the following day.

Hydrodynamic diameter and size distribution of nanoparticles were determined by dynamic light scattering (DLS) measurements, using a Malvern Zetasizer NS (Malvern Instruments, UK); the samples taken over from the reaction mixture after dialysis were sonicated for 3 min. Each sample was measured five times, and average serial data were calculated.

Absorbance variation in 2 g% solutions as a function of samples' temperature (from 20 °C to 65 °C) was measured at 400 nm wavelength, on a Specord 200 Analytic Yena UV–vis spectrophotometer; the temperature of the solution was raised at a constant rate of 0.5 °C min^{-1}.

Fluorescence measurements were performed using pyrene (10^{-6} M in water) as fluorescent probe with a Perkin Elmer fluorimeter. Excitation was fixed at 335 nm, and emission spectra were recorded in the range 350–500 nm. Ratio of emission intensities I_1 (373 nm)/I_3 (383 nm) was calculated and plotted as a function of temperature (Henni-Silhadi et al., 2008).

Dye (RB, BB, B12) loading was determined by a procedure adapted after previously described methods (Dulong, Le Cerf, Picton, & Muller, 2006; Gigimol & Mathew, 2003). The three dyes have different water solubility, but for sake of comparison solution concentrations used in the drug loading experiments were very similar and lower than the solubility limit of the least soluble dye (RB), *i.e.* about 0.1–0.2 g/L. The dry gel (50 mg) was equilibrated with a 50 mL aqueous solution of dye for 48 h, after which solutions containing the nanoparticles and the dye were dialyzed against water to remove the unbounded dye. The amount of bonded dye was determined by UV measurements, reading the absorbance of the dialyzed solutions at specific wavelength: 548 nm (RB), 586 nm (BB) and 550 nm (B12).

Biologically active substances loading was performed as follows: 30 mg nanoparticles were dispersed in 20 mL solutions of drugs with known concentration, and stirred for 48 h. DCF and MB were prepared in water solutions, but ethanolic alpha-tocopherol

Table 1
Physico-chemical characteristics of CMP and oxidized CMP.

Sample	Theoretical DO (molar ratio $NaIO_4$/CMP unit)	DO with CHO groups ($H_2NOH.HCl$ reaction)	M_n [a] (g/mol)	M_w [b] (g/mol)	PDI[c] (M_w/M_n)
CMP	–	–	110,000	250,000	2.3
Ox CMP	0.2	0.34	12,000	19,000	1.6

[a] Number-average molecular weight estimated by SEC.
[b] Weight-average molecular weight estimated by SEC.
[c] Polydispersity index.

solutions were added to the nanoparticles previously hydrated with 5% (related to the total liquid volume) water. The polymer-loaded drug was separated from the solution by centrifugation for 60 min at 12,000 rpm. Supernatant solution was analyzed by UV measurements (DCF: 276 nm; alpha-tocopherol: 292 nm and methylene blue: 668 nm) and the retained drug was calculated with the Eq. (1):

$$\text{Drug retention}\,(\text{g/g}) = \frac{D_i - D_f}{m} \quad (1)$$

where D_i, initial amount of the drug in solution and D_f, final amount of the drug in supernatant; m, nanoparticles amount.

Drug release studies were carried out as follows: 30 mg of drug loaded polymer nanoparticles was dispersed in 3 mL PBS buffer, pH 7.4 in dialysis tubing from cellulose acetate membrane (cut-off: 10,000), using 30 mL PBS as an external solution. At pre-determined time intervals, the outer buffer solution was removed and replaced with an equal volume of fresh buffer. The released drug was determined from UV absorbance of each collected buffer solution.

2.2.3. Antioxidant activity

The antioxidant activity of the alpha-tocopherol released from loaded nanoparticles was determined by the DPPH method (Ara & Nur, 2009) and compared with that of free alpha-tocopherol. Briefly, various amounts of samples containing the antioxidant were immersed in a NaCl aqueous solution (2 mL 0.15 M), then a DPPH solution (1 mL, 0.128 g/L methanol) was added. After 30 min, absorbance of the supernatant at 517 nm was measured. The radical scavenging activity was calculated using the following Eq. (2):

$$\text{Scavenging effect}\,(\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (2)$$

where A_0 is the absorbance of a standard solution prepared under similar conditions, but containing only DPPH, and A_1 is the absorbance of the supernatant. A decreased absorbance of DPPH indicates a higher antioxidant effect.

3. Results and discussion

Reaction of polysaccharides with periodate converts the vicinal dihydroxyl groups (glycol) to paired aldehyde groups. This reaction occurs mainly by scission of C3—C4 and C2—C3 glucosidic bonds, as reported for dextran (Maia et al., 2005) and C2—C3, as reported for CMC (Teotia, 2012), and formation of two aldehyde groups. The oxidation level closely followed the amount of periodate used (with 2 moles of aldehyde groups expected per mole of periodate used). Under such conditions, introduction of CHO groups on the CMP backbone causes chain degradation, as already mentioned in literature for other polysaccharides (Domb, Linden, Polacheck, & Benita, 1996; Gomez, Rinaudo, & Villar, 2007; Liu et al., 2009; Maia, Carvalho, Coelho, Simões, & Gil, 2011). Table 1 presents molecular characteristics of parent CMP and of its oxidized derivative.

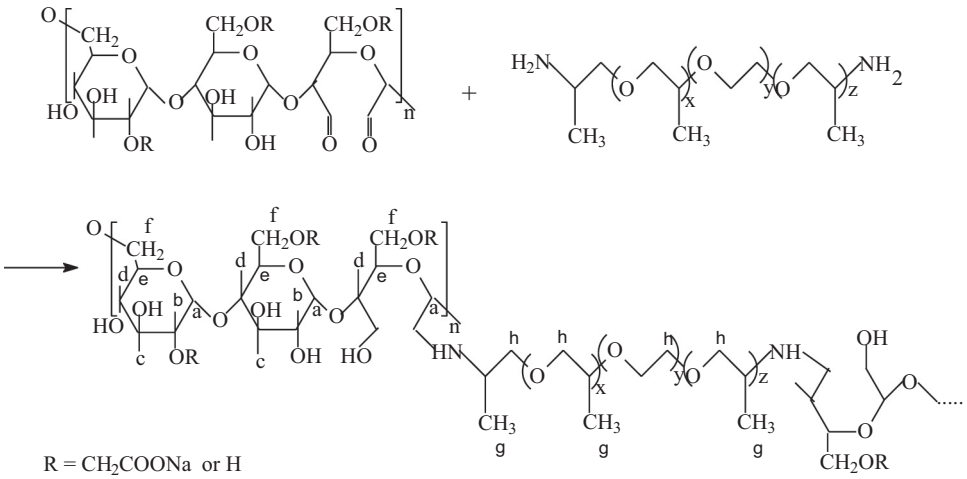
As one see, chain degradation is significant. The molecular weight of the polysaccharide may influence the size of nanoparticles, as reported for hyaluronic acid (Bodnár et al., 2009), but in the case of chitosan the influence was weak (Bodnar et al., 2006).

The reaction between the CHO groups of the CMP and NH_2 groups of Jeffamines performed at various molar ratios Jeff/glucopyranosic unit is presented in Scheme 1.

The reaction takes place mainly intermolecularly (crosslinking), but some intramolecular interactions (cyclization) may occur.

Table 2 presents the reaction conditions and characteristics of the obtained nanoparticles.

The data presented in Table 2 show that the degree of substitution (DS) with Jeff units determined from nitrogen analyses was smaller than the initially amount of Jeff used in reaction.



Scheme 1.

Table 2
Reaction conditions and physico-chemical characteristics of the obtained nanoparticles.

Sample	Jeffamine	Molar ratio Jeff/GU[a]	Nitrogen content, g%[b]	DS Jeff from N content	Average hydrodynamic diameter (DLS), nm (%)[c]
J600: 0.1	ED-600	0.1/1	0.9	0.08	165 ± 10 (82%) 50 ± 7 (18%)
J600: 0.5	ED-600	0.5/1	1.9	0.25	159 ± 20 (89%) 15 ± 5 (11%)
J600: 1	ED-600	1/1	2.2	0.3	159 ± 18 (90%) 4 ± 1 (10%)
J2003: 0.1	ED-2003	0.1/1	0.5	0.06	230 ± 15 (82%) 80 ± 10 (18%)
J2003: 0.5	ED-2003	0.5/1	0.94	0.23	195 ± 18 (83%) 6 ± 2 (17%)
J2003: 1	ED-2003	1/1	1.10	0.29	192 ± 17 (85%) 4 ± 2 (15%)

[a] Glucopyranosic unit.
[b] Determined through EDX – Energy Dispersive X-ray method.
[c] The values are a mean of 4–5 measurements.

The crosslinking degree (determined from nitrogen content of the nanoparticles) depends on the Jeff/glucopyranosic unit molar ratio, but it had a lower influence on the particle dimensions. This lack of crosslinking influence has been already reported in literature for hyaluronic acid nanoparticles (Papadimitriou et al., 2012), but for other hyaluronic acid nanoparticles a decrease of the particle size is reported as the crosslinking ratio increases (Bodnar, Hartmann, & Borbely, 2005). One can mention however that, generally, the nanoparticles crosslinked with Jeff ED600 have smaller dimensions than those obtained with Jeff ED2003. In all cases, the particle size distribution was bimodal. The main population with the diameter ~200 nm results from the intermolecular crosslinking of the oxidized pullulan chain. The second population with small size can be the result of intramolecular reaction of Jeff difunctional derivatives with formation of unimer (single chain) particles. The occurrence of this second population is favored by the high dilution of the reaction medium, and its size decreases with increasing crosslinking degree. Sometimes, several nanoparticle aggregations can be also present, even if in a rather insignificant amount (Fig. 1). Free amino groups, potentially remaining from uncrosslinked Jeff ED, determined by acid orange retention (Fras Zemljic, Strnad, Sauperl, & Stana-Kleinschek, 2009) were not detectables, which means that both amino groups were involved in the crosslinking reaction.

The ^{1}H NMR spectrum of Jeff-CMP nanoparticles in D_2O (Fig. 2) evidences peaks of Jeff CH and CH_2 protons of propylene oxide (PPO) and ethylene oxide (PEO) units, which, together with the peaks corresponding to polysaccharide CMP (CH and CH_2) appear at 3.5–3.7 ppm (b, c, d, e, f, h), and peaks corresponding to the CH_3 protons of PPO units at 1.15–1.3 ppm (g); also, 2 anomeric protons H1–4 and one anomeric proton H1–6 (a) at 5.0–5.7 ppm may be observed; the chemical shift values agree with published results (Dulong, Mocanu, Picton, & Le Cerf, 2012; Glinel, Sauvage, Oulyadi, & Huguet, 2000). Integration of the characteristic peaks of Jeff (methyl protons at 1.15–1.3 ppm, (g) and CMP's anomeric protons at 5.0–5.7 ppm, (a) allowed the calculation of the experimental Jeff content for each sample. The results confirmed those obtained from nitrogen analyses.

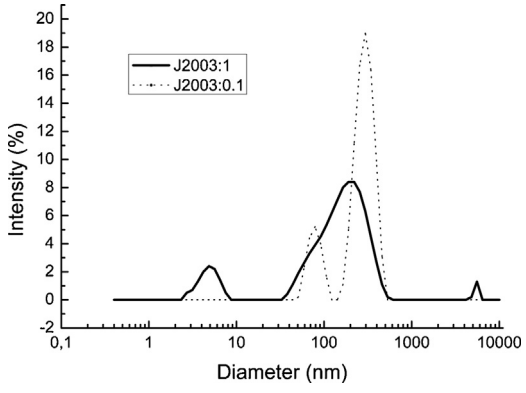


Fig. 1. Size distribution by intensity of J2003 crosslinked nanoparticles.

TEM micrographs (Fig. 3) confirm the nano-size of dried crosslinked CMP-Jeff particles, evidencing a quite narrow distribution of nanoparticle dimensions in dried state and their spherical shape.

Jeffamines are block copolymers with amphiphilic and thermosensitive properties, and the nanoparticles containing them should display similar characteristics. We used different techniques and methods to prove both the amphiphilic and thermosensitive character of synthesized nanoparticle.

3.1. The amphiphilic character

CMP-Jeff nanoparticles have both amphiphilic segments and carboxylic groups, therefore they can strongly interact with other molecules by both hydrophobic and electrostatic forces. The occurrence of such interactions and their preponderence was studied by following the capacity of nanoparticles to retain dyes of different hydrophobic/hydrophilic character. Scheme 2 presents the chemical structures of the used dyes.

Fig. 3 presents dyes adsorption on the CMP-Jeff nanoparticles. RB has a hydrophobic molecule and its retention may be a measure of support hydrophobicity (Gigimol & Mathew, 2003). As seen, RB is retained on the obtained supports in amounts varying between 35 and 55 mg/g. BB is an amphiphilic molecule, having both anionic (sulfonic), and cationic (amine) groups, as well as hydrophobic benzene moieties, consequently, it can interact by both electrostatic forces with amine and carboxylic groups of the supports, and hydrophobic forces with PPO units of Jeff segments The amount of BB retained on the nanoparticles ranges between 140 and 165 mg/g, and depends on the crosslinking degree and Jeff used. B12 is retained through formation of interpolymer complexes, as in cases of complexation with a weak acid cation exchange resin (Mahore, Wadher, Umekar, & Bhoyar, 2010) or with poly(methacrylic acid-g-ethylene glycol) hydrogels (Fogueri & Singh, 2009), but other types of interactions (hydrophobic, hydrogen bonds) can be involved (Mocanu & Nichifor, 2014; Mocanu, Souguir, et al., 2012). B12 is retained by nanoparticles in lower amounts (12–25 mg/g) than the previously discussed dyes. On all supports, dye retention decreases as the crosslinking degree increases, which means that this process is also influenced by the accessibility of the solute inside the macromolecular network. Hence, dyes retention is mainly the result of

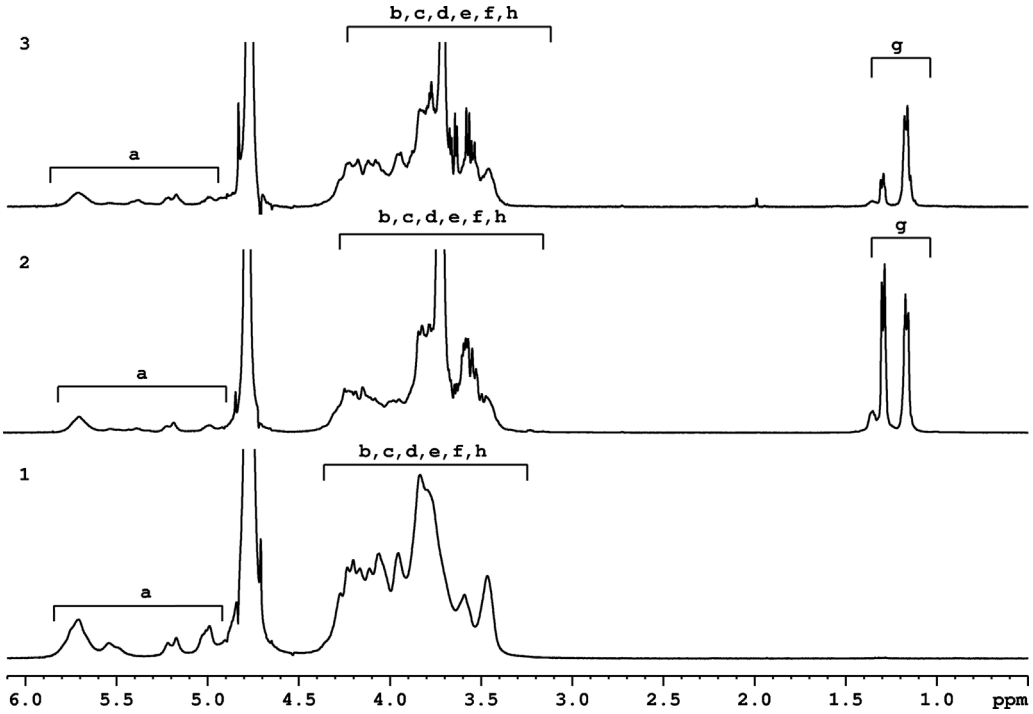


Fig. 2. ^{1}H NMR spectra of CMP (1), J600: 0.5 (2) and J2003: 0.1 (3). The letters on the peaks corresponds to the chemical structure depicted in Scheme 1.

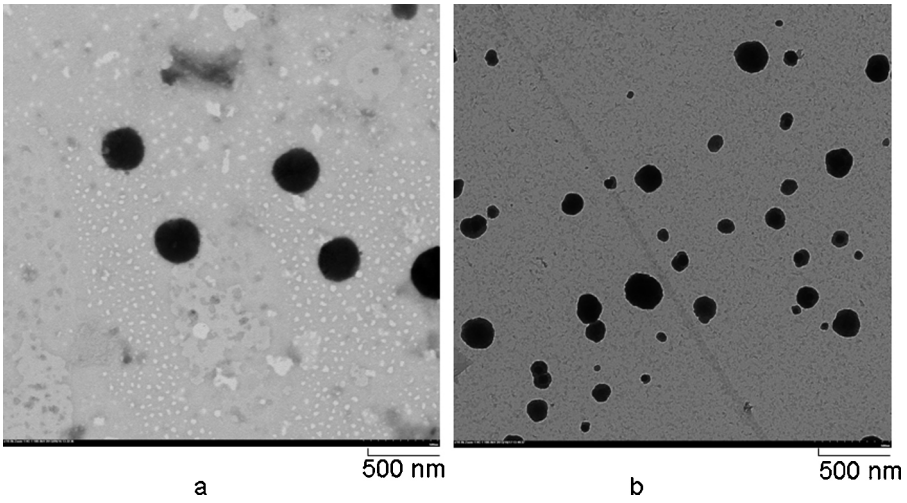
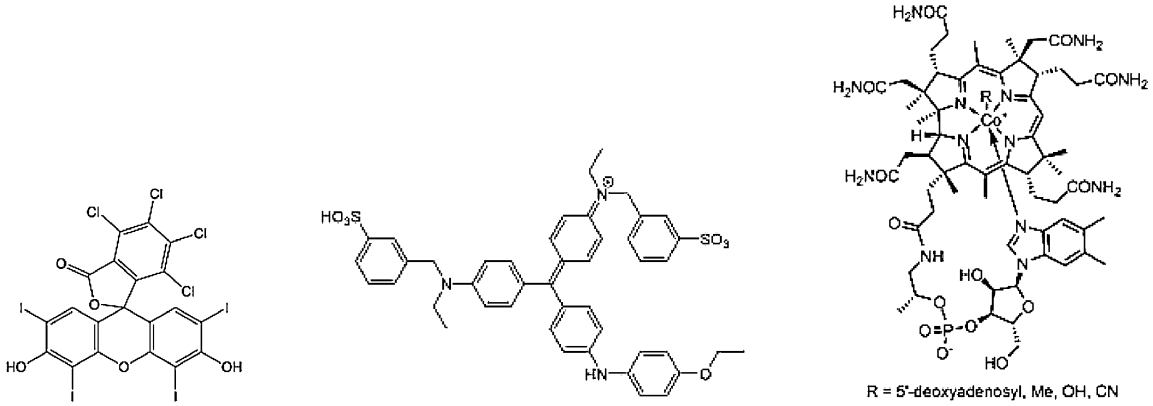


Fig. 3. TEM photos of J600: 0.5 (a) and J2003: 0.1 (b).



Rose Bengal Brilliant blue G Vitamin B12

Scheme 2.

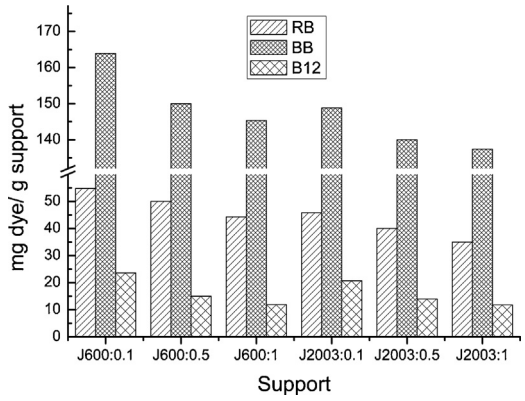


Fig. 4. Dyes retention by the nanoparticles at 20 °C. The values are the mean of three independent measurements with 2–4% standard deviation.

both electrostatic and hydrophobic forces, with some contribution of diffusion factors (Fig. 4).

3.2. Thermosensitive character

The thermosensitive character of the crosslinked CMP-Jeff nanoparticles was evidenced through three methods.

(a) *Absorbance measurements* (at 400 nm wavelength) as a function of temperature. The nanoparticles crosslinked with Jeff ED2003 show an increasing absorbance with temperature increase (in domain 35–40 °C) (Fig. 5a), while those crosslinked with Jeff ED 600 did not (data not shown). Temperature increase is accompanied by reinforcement of the hydrophobic interactions between the hydrophobic segments and weakening of hydrogen bonding with water molecules, which results in the shrinkage of the network, due to the hydrophobic intermolecular interactions. In this context, the particles become more compact and the absorbance increases. At the crosslinking ratios used, this phenomenon is evident for the CMP-Jeff ED2003 nanoparticles. The small variation of the absorbance with temperature is specific to crosslinked thermosensitive polymers, as we have already observed for CMP crosslinked with Jeff *via* amide bonds (Mocanu, Mihai, et al., 2012). By contrary, CMP modified with monofunctional Jeff units (linear, noncrosslinked polymer) displayed a much more significant variation of the absorbance with temperature (Mocanu, Mihai, Dulong, Picton, & Le Cerf, 2011).

(b) *DLS measurements*. Variation of nanoparticles' size as a function of temperature was highlighted through DLS measurements. The data presented in Fig. 5b show that microparticles' size decreases as temperature increases from 25 to 40 °C; the size decrease is more pronounced for the more crosslinked nanoparticles possessing thermosensitive Jeff 2003 units.

(c) *Fluorescence measurements.* The thermosensitive character of the CMP-Jeff nanoparticles was evidenced also by the variation of I_1/I_3 values in the pyrene emission spectrum as a function of temperature (Fig. 5c). The measurements were carried out on the J2003: 1 sample. The decrease of the I_1/I_3 values with temperature increase is specific to thermoresponsive polymers (Feil, Bae, Feijen, & Kim, 1993) and can be explained by intrapolymeric aggregation, resulting in the formation of a phase that provides a hydrophobic environment where pyrene can be sequestered (Liaw, Huang, & Wang, 2002). Monotonous and less pronounced decrease of I_1/I_3 values was observed for other crosslinked CMP-Jeff thermosensitive polymers (Mocanu, Mihai, et al., 2012), unlike to linear, uncrosslinked CMP-Jeff derivatives, where I_1/I_3 variation with temperature has a break point and is more significant (Mocanu et al., 2011).

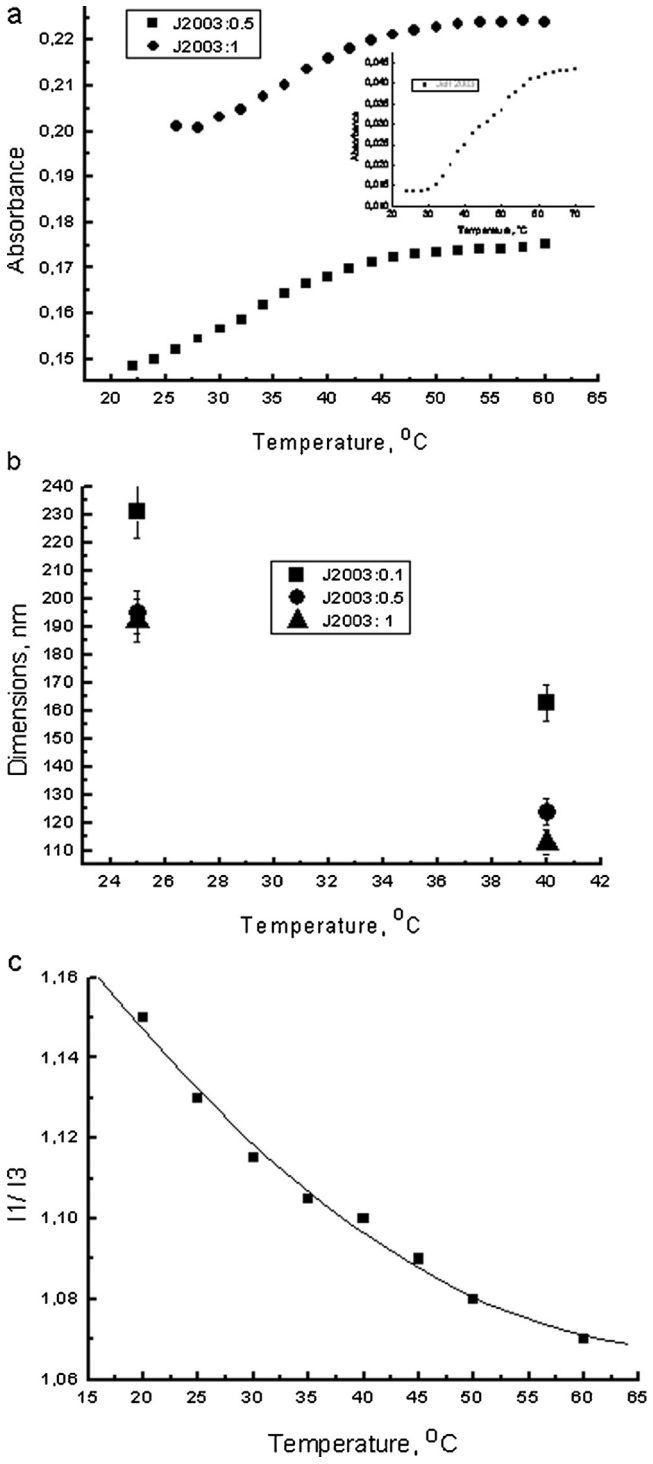


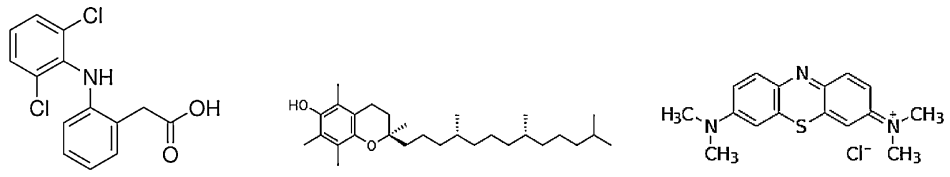
Fig. 5. Absorbance (a), size (b) and I_1/I_3 ratio (c) variation with temperature. The values are the mean of three independent measurements with 2–4% standard deviation.

3.3. Interaction with biomolecules

3.3.1. Drug retention

The interaction of the obtained nanoparticles with anionic (DCF), cationic (MB) and uncharged (α-TF) biologically active substances was studied to evaluate their potential for controlled drug release systems.

Their formula is presented in Scheme 3.



Diclofenac (DCF) alpha Tocopherol (α-TF) Methylene blue (MB)

Scheme 3.

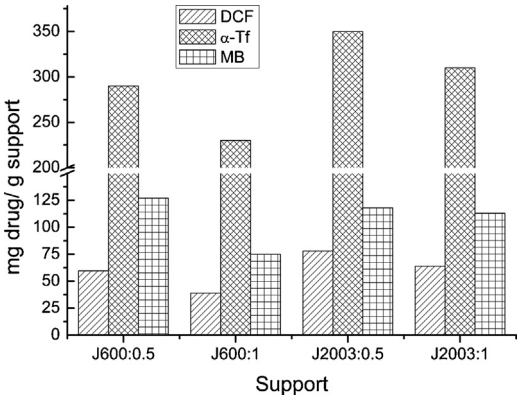


Fig. 6. Drug retention by the nanoparticles; the values are the mean of three independent measurements that deviated: 2–5%.

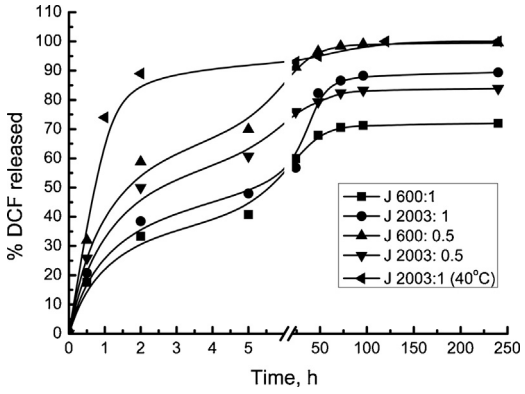


Fig. 7. *In vitro* release of DCF retained by the nanoparticles; the values are the mean of three independent measurements that deviated: 2–4%.

DCF [2-(2,6-dichloranilino) phenylacetic acid] is a nonsteroidal anti-inflammatory drug used to reduce inflammation and also as an analgesic for reducing pain in certain conditions. Due to its anionic character, it can be retained on CMP-Jeff nanoparticles mainly through electrostatic forces, on the secondary aminic groups formed as a result of crosslinking with di-amino Jeffamine units.

Tocopherols are lypophilic antioxidants with vitamin E activity. α-TF is the most biologically active form of vitamin E, that is preferentially absorbed and accumulated in humans. Being fat-soluble, it is incorporated into cell membranes, protecting them against oxidative damage. α-TF has a regulatory effect on either enzymatic activity or gene expression and plays a role in neurological functions. Its retention on the synthesized nanoparticles can be attributed essentially to the hydrophobic forces.

MB presents different biologically activities as a function of the administrated dose. At low doses (micro-gram level) improvements were observed in memory consolidation, along with is a neuroprotective effect. It also has urinary analgesic/anti-infective/anti-spasmodic effects. Due to its basic character, it may be retained on CMP-Jeff nanoparticles by electrostatic interactions with carboxylic groups.

According to the data presented in Fig. 6, all supports retain important amounts of drugs, and the retention capacity decrease in the order α-TF > MB > DCF, indicating that hydrophobic interactions play an important role. The loading capacity depends on support chemical structure. Thus, nanoparticles crosslinked with Jeff ED2003 retained higher amounts than the ones crosslinked with Jeff ED600, and this influence is more significant for α-TF. The supports with lower crosslinking degree retain higher drug amounts, hence one can appreciate that, besides the hydrophobic forces, drug retention is also influenced by a diffusion process inside the macromolecular network. DCF is retained in higher amounts on less crosslinked supports, even if they have less aminic groups formed as a result of crosslinking, showing that in this case diffusion factors predominates over electrostatic forces. MB is retained through electrostatic forces on the carboxymethylic groups of the supports. Since all supports contain the same amount of carboxylic groups, MB retention should be approximately the same, but the diffusion process might have some influence, too.

3.3.2. Drug release

DCF release (Fig. 7) occurs gradually; the less crosslinked nanoparticles: J600: 0.5 and J2003: 0.5 release the drug faster than more crosslinked ones: J600: 1 and J2003: 1. An initial fast release of DCF (about 18–32%) was observed along a 30 min period. This initial rapid release, characterized as a "burst effect", is due to the fact that some amounts of DCF were localized on the surface of nanoparticles through adsorption and could be easily released. After this initial burst effect, a slower sustained and controlled release occurred throughout the incubation period, the release amount being 72–99.5%. At 40 °C DCF is released much faster, about 70% being released in 1 h. This behavior is determined by the network shrinking as temperature increase, alongside the "burst effect", and confirms once more the thermosensitive properties of the support.

Hence, the obtained nanoparticles can be considered as valuable supports for controlled release drug systems.

α-TF in vitro release was tested under the same conditions used for DCF. The data presented in Fig. 8a show that the more hydrophobic drug is released very slow at 20 °C, perhaps due to the strong hydrophobic interactions of the drug with the support and/or to the smaller solubility of the drug in PHB buffer. Also, as in case of DCF, releasing rate increased by raising the temperature at 40 °C, which can be explained by temperature effect on both network shrinking and drug solubility in PHB. At both temperatures, the drug is faster released from the less crosslinked support (J2003: 0.5 toward J2003: 1).

Antioxidant activity. In order to know if α-TF retained by the supports still presents antioxidant activity, this characteristic (expressed as Scavenging effect %) of the supports containing α-TF was studied comparatively with that of the drug itself. The data presented in Fig. 8b reveal that α-TF retained by the studied supports show antioxidant activity. The scavenging effect is

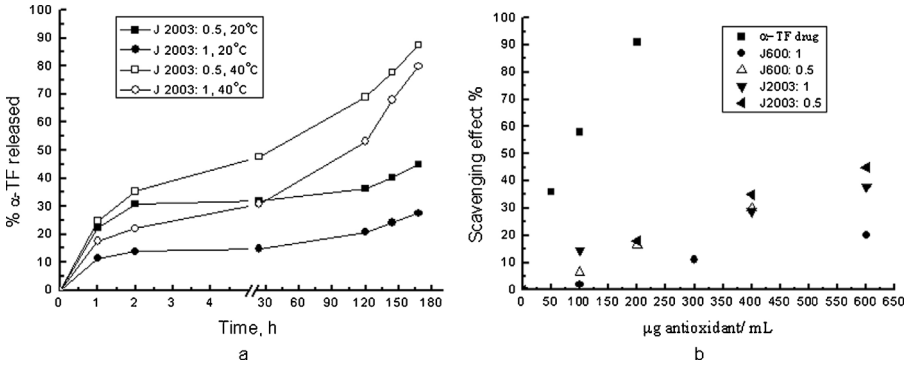


Fig. 8. a) *In vitro* release of α-TF retained by the nanoparticles; the values are the mean of three independent measurements with 4% standard deviation; (b) Scavenging effect of α-TF loaded nanoparticles, comparatively with that of α-TF itself; the concentration, in μg/mL represents the amount of α-TF contained by the nanoparticles, reported to the solution volume. The values are the mean of three independent measurements with 3% standard deviation.

produced only by the released α-TF, and the lower values observed in the presence of drug-loaded nanoparticles compared with parent α-TF, indicate a slow and gradual release of the drug retained by supports. This behavior can be important in the potential use of supports for controlled delivery of antioxidant substances.

4. Conclusions

CMP nanoparticles were synthesized through the reaction of oxidized CMP with difunctional Jeffamines, in dilute aqueous solutions, at room temperature. Their structure was confirmed by ^{1}H NMR. The dimensions were measured by DLS, while TEM technique confirmed the nano-size of the dried crosslinked CMP-Jeff particles and their spherical shape. The amphiphilic character of the nanoparticles was evidenced through amphiphilic, hydrophobic and hydrophilic dyes retention. Their thermosensitive character was evidenced through variation of absorbance, size and pyrene fluorescence. The retention/release of some acidic (diclofenac), hydrophobic (α-TF), basic (methylene blue) drugs, as well as determinations of the antioxidant activity (expressed as scavenging effect) prove perspectives for the application of these nanoparticles as supports for controlled release drug systems. Due to their property to retain metals (on aminic functions) possible applications in bio-medical domain to target biologically active substances or for diagnosis purposes also can be envisaged.

Further *in vitro* and *in vivo* studies are required for ascertaining the biological potential of these new nanoparticles.

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