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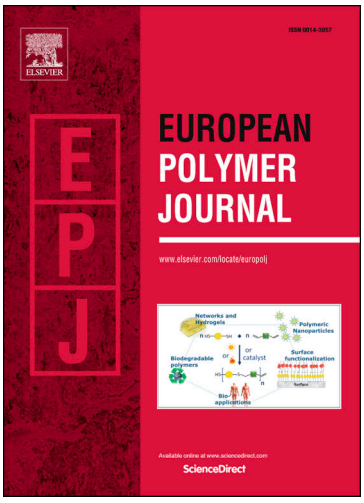

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Polysaccharide-fullerene supramolecular hybrids: synthesis, characterization and antioxidant activity

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Keywords: Hydrophobized polysaccharides; Cholesterol-levan conjugate; Polysaccharide-C_{60} nanoparticles; DLS; SEM; Antioxidant activity

Abstract

An efficient encapsulation of the fullerene into two hydrophobized and one native polysaccharide provided water soluble supramolecular hybrids. After covalent modification of polysaccharides by cholesterol, noncovalent hybrids were prepared by a three-step procedure, including mixing of individual aqueous solutions of hydrophobized, as well as native sugar with solution of the fullerene in pyridine, dialysis and lyophilization. Although the degree of the fullerene incorporation into hydrophobized substrates, cholesterol-levan and cholesterol-pullulan, was lower in comparison to the native polysaccharide levan, hydrophobization provided nanoparticles with improved properties. The particle size distribution, studied by dynamic light scattering and scanning electron microscopy revealed formation of moderately polydisperse aggregates, with the diameter contraction in comparison to the corresponding free polysaccharide, especially in the case of hydrophobized substrates. The morphological examination, done by scanning electron microscopy indicated the self-organization of the fullerene-native polysaccharide to round individual structures, while fullerene-hydrophobized polysaccharide hybrids tend to form networks. The antioxidant activity of the synthesized polysaccharide-C_{60} noncovalent hybrids versus starting polysaccharides was investigated by the DPPH radical scavenging and the β-carotene-linoleic acid bleaching methods. In all three complexes, the radical scavenging ability of the fullerene remained preserved, and a positive effect of levan hydrophobization was observed.

1. Introduction

The fullerene C_{60} exhibits extraordinary photochemical, photophysical, and electrochemical properties, making itself a very attractive building block for the construction of different structures with a wide range of potential applications, especially in materials science [1,2] and medicinal chemistry [3-5]. Therefore, fullerene, as a strongly hydrophobic nanomaterial essentially insoluble in water and other polar media [6], represents a tempting structure in the synthetic sense, in aim to increase its solubility in aqueous media and expanding its biological application. Two main approaches for functionalization of the fullerene core, covalent and supramolecular, have been developed. Next to covalent fullerene chemistry [5], the noncovalent functionalization of fullerene C_{60} with water-soluble carriers that can form host–guest complexes represents a fast-developing strategy for the preparation of C_{60}-based hybrid nanomaterials. Supramolecular incorporation of C_{60} in special carriers like cyclodextrins [7-12], calixerenes [13], biocompatible polymers, such as polyvinylpyrrolidone [14,15], poly(2-(methacryloyloxy)ethyl phosphorylcholine [16], PEG [17], and poly(2-oxazoline)s [18,19], as well as liposomes [20,21], proteins [22], and antibiotic doxorubicin [23], gives soluble complexes without changing the physical properties of the fullerene sphere. In addition, increasingly complex molecular systems for solubilization and studies of fullerene were used. Murthy et al. [24] reported the synthesis of a water-soluble

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encapsulated polymer, poly(β-cyclodextrin-lactose)/C_{60} inclusion complex, starting from a lactose-based polymer. This supramolecular conjugate exhibits higher water solubility compared with that of unmodified β-cyclodextrin/C_{60}, and possesses radical scavenging activity in a preliminary study using 2,2'-diphenyl-1-picrylhydrazyl (DPPH) test, pointing to possible biomedical application. Fumitoshi and coworkers [25] have reported use of human serum albumin (HSA) as a dissolution agent and the formation of stable C_{60}/HSA nanoparticles via soluble C_{60}/2-hydroxypropyl-modified β-cyclodextrin nanoparticles. C_{60}/HSA nanoparticles with a small dispersion size showed a high antioxidant activity and significant phototoxic properties, thus creating a possible drug delivery system for photodynamic therapy. Liu and coworkers [26] designed and synthesized water-soluble polysaccharide–porphyrin–C_{60} supramolecular conjugates, starting from porphyrin-modified β-cyclodextrins, adamantyl-modified polysaccharide hyaluronate, and C_{60}. These supramolecular complexes, existing as nanoparticles with a diameter of 50–200 nm, exhibited good DNA cleavage activity under visible light irradiation, expanding the potential of modified polysaccharide-based supramolecular nanoparticles for pharmaceutical and biological applications. Another way of formation of aqueous dispersions of the fullerene C_{60} by simple mixing of the solution of C_{60} in *N*–methylpyrrolidone with aqueous solution of natural molecules, such as *L*-amino acids, monosaccharides, or peptides as stabilizing agents followed by dialysis method has been studied by Andreev et al. [27]. Also, naturally and commercially available disaccharides (lactose, maltose and sucrose) were used for transfer of the C_{60} molecules into water by the eco-friendly procedure for preparation of complexes with improved solubility and stability compared to the C_{60}-cyclodextrin inclusion complex [28]. The morphology, particle size, and radical scavenging properties of lactose-C_{60} nanoparticles in water were studied by transmission electron microscopy (TEM), static light scattering (SLS) and DPPH assay, respectively, indicating that aqueous lactose-C_{60} spherical nanoparticles of ~60 nm in size could be used as an antioxidant. Litvinova and colleagues used mechanochemically stimulated synthesis to solubilize C_{60} by sucrose and dextran and found that sucrose had higher solubilizing potential than dextran [29]. Performed studies of the stability of fullerene-carbohydrates composites in water suggested that the solubilization of the fullerene was achieved by formation of van der Waals complex of fullerene aggregates with the carbohydrate shell.

Based on the aforementioned studies on supramolecular nanomaterials containing C_{60} and different carbohydrates (lactose, maltose, sucrose, dextran and hyaluronic acid), developed by several research groups [24,26,28,29], the complexing of C_{60} with non-toxic, biocompatible, and biodegradable natural carbohydrates and their derivatives, was proved to be a valuable strategy to generate C_{60}-based hybrid nanoparticles, but with insufficient information on their biological properties. Because of its unique structure, polysaccharide pullulan and its derivatives are attractive target molecules with a great potential for applications in diverse scientific fields, such as biomedical, pharmaceutical, and food sciences [30,31]. Pullulan is a water-soluble linear homopolysaccharide of D-glucose which contains the α-1→6-connected maltotriose repeating units (Glc-α-1→4-Glc-α-1→4-Glc) with nine OH groups which can easily be chemically modified. Cholesterol-pullulan conjugates [32,33] with different substitution degree are among the most studied hydrophobized polysaccharides for developing self-aggregated monodispersed nanoparticles, in which, hydrophilic pullulan chains are self-assembled in water thanks to the hydrophobic interactions of grouped cholesteryl moieties. Cholesterol-pullulan, as a hydrophobically modified amphiphilic polymer, is a good carrier for other hydrophobic molecules, such as a peptide hormone insulin [34], an anticancer drug mitoxantrone [35], β-amyloid oligomers [36], a natural alkaloid vincristine [37], as well as for fullerene C_{60} [38]. The Sunamoto group [35] reported a synthesis of a water-soluble complex between cholesteryl-hydrophobized polysaccharide pullulan and C_{60} and characterization of the obtained nanoparticles by dynamic light scattering (DLS) and cryo-TEM. Unlike polydisperse free C_{60} aggregates, the size of nearly monodisperse self-aggregated nanoparticles of this complex in PBS buffer was in the range of 60-150 nm. It is necessary to emphasize that the aqueous solution of the complex was stable for several months, as well as after lyophilization. In contrast to this successful synthesis with modified pullulan, the corresponding complex with unmodified pullulan was not obtained under the same conditions. Their results show that it is possible to prepare stable C_{60}-hydrophobized pullulan nanoparticles in aqueous media, which could have potential as antioxidants owing to the potent radical scavenging ability of the unmodified fullerene [39,40]. Inspired by these promising studies and, on the other hand, very scarce information on applicability of this complex, we have decided to expand our initial research concerning the synthesis of cholesterol-hydrophobized pullulan to another natural polysaccharide levan, its hydrophobization, and finally the preparation of their supramolecular hybrids with C_{60}. In addition to the general characteristics, a water soluble levan, a β-2,6-linked fructose polymer, possesses enhanced flexibility compared to the other linear polysaccharides, as well as the variety of current and potential applications ranging from medical and pharmaceutical uses to the food industry [41,42]. To date there is no studies on the hydrophobization of levan with a steroid moiety, nor preparation of fullerene-containing levans.

The aim of presented study was an improvement of fullerene solubilization by noncovalent modification, supported by easily available and biologically tolerant carrier levan. At the same time, attenuation of the hydrophilic character of polysaccharide should facilitate interaction with strongly hydrophobic fullerene, so the whole study was performed using the native levan and its hydrophobized analog, together with hydrophobized pullulan as a reference compound.

Native polysaccharides were covalently hydrophobized with cholesterol, over hexamethylene dicarbamate linker, using the procedure described in the literature [33]. In such a way, already studied cholesterol-pullulan (CHP) conjugate, and the new one – cholesterol-levan (CHL) were obtained and used as substrates in preparation of supramolecular hybrids with C_{60}. In addition, for comparison purposes levan-fullerene (L-C_{60}) was prepared and studied. The presence of polysaccharides (PS) in PS-C_{60} hybrids significantly improved solubility of C_{60} in water, enabling the investigation of their antioxidant activity in aqueous media using DPPH radical scavenging and β-carotene-linoleic acid bleaching *in vitro* assays. To gain a more detailed insight into the structure of the obtained material, the particle size distribution and self-assembled characteristics in aqueous solution were also examined using DLS and scanning electron microscopy (SEM).

2. Experimental part

2.1. Materials and methods

Cholesterol (≥99%), 1,6-hexyldiisocyanate (≥99%, GC), linoleic acid (≥99%), Tween 40 (Approximately 90% Palmitic Acid, GC), β-carotene (≥93%, UV), ascorbic acid (European Pharmacopoeia (BP) Reference Standard), 2,6-di-*tert*-butyl-4-methylphenol (BHT) (≥99.0%, GC), and 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) (Product Number D9132) were purchased from Sigma Aldrich and used without further purification. Pullulan (food additive grade, M_w ~10^5, viscosity 132 cm^2/s, purity > 90% on the dry weight basis) was purchased from Hayashibara Co., Ltd, Japan and used without further purification. Levan LS1 (M_w > 10^6, viscosity 0.25 dL/g, purity > 97% on dry weight basis) produced by *B. licheniformis* NS032 was used. Fermentation conditions and purification procedures were described in detail previously [43]. Cholesteryl *N*-(6-isocynatohexyl)carbamate, hydrophobized polysaccharides, CHP-10^5/5.0 and CHL-10^6/1.8, were synthesized according to the procedures reported by Akiyoshi et al. [33] with slight modifications. The label of hydrophobized polysaccharides (CHP-10^5/5.0 and CHL-10^6/1.8) indicates the M_w of the parent polysaccharide and the number of steroid units introduced per 100 monosaccharide units, respectively. IR spectra (ATR in the solid state) were recorded with a Perkin–Elmer-FTIR 1725X spectrophotometer; *v* values are given in cm^{-1}. ^1H and ^{13}C NMR spectra were recorded in the indicated solvent with Bruker Avance (^1H at 500 MHz, ^{13}C at 125 MHz) spectrometer. Chemical shifts (δ) are expressed in ppm and coupling constants (*J*) in Hz. The following abbreviations were used for signal multiplicities (s = singlet, br s = broad singlet, d = doublet, br d = broad doublet, t = triplet, br q = broad quartet, m-multiplet, St-steroidal, h-hexyl, P(A,B,C) corresponds to a maltotriose unit: Glc-α-(1,4)-Glc-α-(1,4)-Glc-α-(1,6), L-levan). The homonuclear 2D (DQF-COSY) and the heteronuclear 2D ^1H-^{13}C spectra (HSQC, HMBC) were recorded with the usual settings. UV spectra were recorded with a Shimadzu UV-1280 UV-Vis spectrophotometer. Dialysis was performed against distilled water using a Membra-cell MD34 14x100CLR molecular dialysis membrane (M_w cut-off 14000 Da).

Size exclusion chromatography (SEC): Samples of polysaccharides, native and modified, were analyzed on the HPLC – AKTApurifier system (GE Healthcare, Germany), equipped with 80 mL Toyopearl HF55 gel exclusion column and UV detector. Flow rate was 1mL/min. The sample was prepared by dissolving in the eluent (water for levan/CHL, 10% ethanol in water for pullulan/CHP) and filtered before injection through a 0.45 μm filter (SM1: Fig. S1).

Dynamic light scattering (DLS) technique was performed on a Zetasizer Nano ZS (Malvern Instruments, UK) in order to investigate the hydrodynamic particle size and particle size distribution (PSD) (diameters) of PS (c(PS)=0.05 mg/mL) and corresponding PS-C_{60} retentates (c(PS+C_{60}) 0.03-0.06 mg/mL). The samples were filtered through a 0.45 μm syringe filter prior to measurements. The PSD results were averaged from at least 3 conducted measurements (10 runs each). The Zetasizer was operated using He–Ne laser (633 nm) and equipped with 173° angle backscattering detection optics.

Scanning electron microscopy (SEM): Investigations of sample morphology were carried out with SEM, using a JEOL JSM-840A instrument, at an acceleration voltage of 30 kV. For SEM imaging, 5 μL of a retentate of the corresponding sample was dropped on the surface of glass substrate and left for 24 h to slowly evaporate at the room temperature. The investigated samples were gold sputtered in a JFC 1100 ion sputter and then subjected to SEM observations.

2.2. Cholesteryl *N*-(6-isocyanatohexyl)carbamate

Cholesterol (0.780 g, 2.02 mmol) was reacted with 1,6-hexyldiisocyanate (5.05 g, 4.86 mL, 30 mmol) in 20 mL of dry toluene containing 0.4 mL of dry pyridine at 80 °C in inert Ar atmosphere for 26 h. The reaction progress was monitored by TLC. After the solvent was removed *in vacuo,* 140 mL of *n*-hexane (HPLC) was added dropwise to the residue (with strong stirring) and it was stored over the weekend at −30 °C. After filtration and drying in vacuo (40 °C, 20 mbar, 12 h), cholesteryl carbamate CHI (550 mg, 49 %) was obtained as a white powder. M.p. 93.0-94.1 °C; IR: 3336, 2939, 2867, 2359, 2258, 1690, 1543, 1467, 1374, 1253, 1138, 1023 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): 5.37 (br d, *J* 5.0 Hz,

1H, HC(6)St), 4.58 (br s, 1H, NHCOO), 4.49 (m, 1H, HC(3)St), 3.30 (t, *J* 6.5 Hz, 2H, $H_2C(6)^h$-NCO), 3.16 (br q, *J* 6.0 Hz, 1H, $H_2C(1)^h$-NHCOO), 1.01 (s, 3H, $CH_3(19)^{St}$), 0.91 (d, *J* 6.5 Hz, 3H, $CH_3(19)^{St}$), 0.87 and 0.86 (two d, *J* 6.5 Hz, 6H, $CH_3(26,27)^{St}$), 0.68 (s, 3H, $CH_3(18)^{St}$); ^{13}C NMR (125 MHz, $CDCl_3$): 156.3 (OCONH)), 140.0 (C(5)St), 122.6 (C(6)St), 122.1 (NCO), 74.4 (C(3)St), 56.8 (C(14)St), 56.3 (C(17)St), 50.2 C(9)St), 43.0 ($H_2C(6)^h$-NCO)), 42.5 (C(13)St), 40.9 ($H_2C(1)^h$-NHCOO), 39.9 (C(12)St), 39.7 (C(24)St), 38.7 C(4)St), 37.2 (C(1)St)), 36.7 (C(22)St), 36.3 (C(10)St), 36.0 (C(20)St), 32.0 (2C, C(7)St, C(8)St), 31.3 ($CH_2(5)^h$), 30.1 ($CH_2(2)^h$), 28.4 (C(2)St), 28.3 (C(16)St), 28.2 (C(25)St), 26.4 ($CH_2(4)^h$), 26.3 ($CH_2(3)^h$), 24.4 (C(15)St), 24.0 (C(23)St), 23.0 and 22.7 ($CH_3(26,27)^{St}$), 21.2 (C(11)St), 19.5 ($CH_3(19)^{St}$), 18.9 ($CH_3(21)^{St}$), 12.0 (C(18)St).

2.3. Synthesis of hydrophobized polysaccharides

2.3.1. Cholesterol-substituted pullulan (CHP)

Cholesteryl *N*-(6-isocyanatohexyl)carbamate (0.034 g, 0.062 mmol) was reacted with pullulan (0.400 g, 2.47 mmol as the glycoside unit, pullulan was vacuum-dried for at least 2 days at 50 °C) in 10 mL of dry DMSO containing 0.80 mL of pyridine at 80 °C for 8 h in the presence of Ar. Ethanol (70 mL) was added to the reaction mixture, and the obtained suspension was stored overnight at 4 °C. The precipitates were separated, purified by dialysis against water and lyophilized to give white powder (0.214 g, 46%). The degree of substitution (DS) of the cholesterol group per 100 glucose units in pullulan was determined by the ^{1}H NMR spectrum (Fig. 2A). The primary hydroxyl groups of pullulan (M_w ~10^5) were substituted by 5.0 cholesterol units per 100 glucose monomers (denoted as CHP-10^5/5.0).

IR: 3353, 2930, 1648, 1419, 1150, 1080, 1024 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): 5.55 (br s, 2OH^P), 5.42 (br s, OH^P), 5.39 (br s, OH^P), 5.33 (br s, HC(6)St), 5.03 (br s, HC(1)$^{P(B)}$), 4.99 (br s, HC(1)$^{P(A)}$, 3OH^P), 4.71 (br s, OH^P), 4.67 (br s, HC(1)$^{P(C)}$), 4.46 (br s, OH^P), 4.29 (m, HC(3)St), 3.89 and 3.51 (two br s, $H_2C(6)^{P(C)}$), 3.71 and 3.51 (two br s, $H_2C(6)^{P(A)}$), 3.62 (br s, $H_2C(6)^{P(B)}$), 3.40-3.00 (br s, HC(2-5)^P), 0.96 (s, $CH_3(19)^{St}$), 0.89 (d, 6.0 Hz, $CH_3(21)^{St}$), 0.84 (d, *J* 4.5 Hz, $CH_3(26,27)^{St}$), 0.65 (s, $CH_3(18)^{St}$); ^{13}C NMR (125 MHz, $CDCl_3$): 155.8 (OCONH)), 121.7 (C(6)St), 72.8 (C(3)St, from HSQC), 101.4 (C-1$^{P(A)}$), 100.7 (C-1$^{P(B)}$), 98.7 (C-1$^{P(C)}$), 80.8 (C-4$^{P(C)}$), 80.0 (C-4$^{P(B)}$), (73.4, 73.3, 73.0, 72.4, 72.3, 71.9, 71.6, 70.7, 70.0 (C-(2-5)^P), 67.0 (C-6$^{P(A)}$), 60.8 (C-6$^{P(C)}$), 60.3 (C-6$^{P(B)}$), 22.7 and 22.4 ($CH_3(26,27)^{St}$), 19.0 ($CH_3(19)^{St}$), 18.6 ($CH_3(21)^{St}$), 11.7 (C(18)St).

2.3.2. Cholesterol-substituted levan (CHL)

Cholesteryl *N*-(6-isocyanatohexyl)carbamate (0.034 g, 0.062 mmol) was reacted with levan (0.400 g, 2.47 mmol as the fructose unit, levan was vacuum-dried for at least 2 days at 50 °C) in 10 mL of dry DMSO containing 0.80 mL of pyridine at 80 °C for 8 h in the presence of Ar. Ethanol (50 mL) was added to the reaction mixture, and the suspension so obtained was stored overnight at 4 °C. The precipitates were separated (480 mg of crude product), purified by dialysis against distilled water and lyophilized to give white powder (0.370 g, 87%). The degree of substitution of the cholesterol unit was determined by ^{1}H NMR (Fig. 2A). The primary hydroxyl groups of levan (M_w >10^6) was substituted by 1.8 cholesterol groups per 100 monomeric units (denoted as CHL-10^6/1.8).

IR: 3338, 2934, 1690, 1416, 1332, 1259, 1124, 1018, 926 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): 5.33 (br s, HC(6)St), 5.14 (br s, OH-C(4)^L), 4.72 (br s, OH-C(1)^L), 4.64 (br s, OH-C(3)^L), 4.29 (m, HC(3)St), 3.98 (br s, HC(3)^L), 3.78 (br s, HC(4)^L), 3.70 (br s, HC(6)^L), 3.62 (br s, HC(5)^L), 3.44 (br s, HC(1,6)^L), 3.34 (br s, HC(1)^L), 0.96 (s, $CH_3(19)^{St}$), 0.89 (d, 5.0 Hz, $CH_3(21)^{St}$), 0.84 (d, *J* 4.5 Hz, $CH_3(26,27)^{St}$), 0.65 (s, $CH_3(18)^{St}$); ^{13}C NMR (125 MHz, DMSO-d_6): 122.4 (C-6)St, from HSQC)), 104.9 (C-2)^L), 80.9 (C-5)^L), 76.6 (C-3^L), 76.0 (C-4^L), 63.6 (C-6^L), 61.3 (C-1^L), 23.3 and 23.0 ($CH_3(26,27)^{St}$), 19.7 ($CH_3(19)^{St}$), 19.2 ($CH_3(21)^{St}$), 12.3 ($CH_3(18)^{St}$).

2.4. Preparation of polysaccharide-C_{60} supramolecular hybrids (in a 2.4:1 weight ratio)

Levan or hydrophobized levan/pullulan (CHL and CHP) (6 mg) was suspended in 120 mL of distilled water and stirred at 55 °C over 72 h. After that, a pyridine solution of fullerene (5 mL, 0.5 mg/mL) was added into an aqueous suspension of polysaccharide at room temperature. The resulting mixture was stirred for 72 h at room temperature in the dark. The solution was dialyzed against distilled water for 3 days to eliminate pyridine. The distilled water was exchanged every day. The polysaccharide-C_{60} retentates were lyophilized to give brownish supramolecular hybrids, L-C_{60} (6.6 mg, 77.6%), CHL-C_{60} (7.0 mg, 82.3%), and CHP-C_{60} (3.4 mg, 40.0%). The lyophilized samples are stored in the dark at room temperature. Their IR spectra are given in SM2 (pp. 36-39). The C_{60} content in C_{60}-polysaccharide retentates was spectroscopically determined in their toluene extracts (Table 1, SM1: Table S1). In addition, weight percentages of C_{60} in solid PS-C_{60} formulations are presented in Table 1 (amount of C_{60}, calculated from concentrations and total volumes of lyophilized solutions, per measured freeze-dried PS-C_{60} powders). Several batches of the PS-C_{60} supramolecular hybrids have been prepared. The biological studies were done on samples obtained from batches with the fullerene content presented in Table 1 and Table S1 (SM1). The stability of PS-C_{60} retentates was monitored for 4 months by recording the UV-spectra (SM1: Fig. S2).

2.5. DPPH Antioxidant assay

DPPH stock solution in methanol (0.27 mM, 0.109 mg/mL) was freshly prepared and consumed in course of the next few hours. DPPH probe was prepared by mixing equal volumes of deionized water, methanol and DPPH stock solution (400 μL : 400 μL : 400 μL).

Blank probes of the samples, with the same dilutions, but with methanol instead of DPPH solution were prepared and UV-vis spectra were recorded.

Dialyzed solutions of PS-C_{60} (400 μL) were strongly shaken with DPPH stock solution (400 μL) and methanol (400 μL) and kept in dark, at room temperature (c=0.017, 0.018 and 0.0056 mg/mL for L-C_{60}, CHL-C_{60} and CHP-C_{60}, respectively).† UV-vis spectra were collected during next four days (at the beginning of the reaction hourly, then once a day for the next three days) (SM1: Fig. S3).

Solid lyophilized PS-C_{60} (L-C_{60}, CHL-C_{60} and CHP-C_{60}) were vigorously stirred in deionized water (1.0 mg/mL) at room temperature, over 24 hours, protected from light, to give stock suspensions for radical scavenging assay. DPPH stock solution (400 μL) and methanol (400 μL) were mixed with strong shaking with four different concentrations of each sample (400 μL of each solution: 1.00 mg/mL, 0.50 mg/mL, 0.25 mg/mL and 0.125 mg/mL), to give final concentration of DPPH 0.09 mM (0.036 mg/mL) and final concentrations of samples 0.333 mg/mL, 0.167 mg/mL, 0.084 mg/mL and 0.042 mg/mL. Probes were kept at room temperature protected from light. UV-vis spectra were monitored during the next 3 hours (SM1: Figs S3 and S4).

Radical scavenging activity (RSA) was expressed as a percentage of the used DPPH after two hours of reaction, performed in dark at room temperature, calculated by Eq. (1).

$$\text{RSA (\% DPPH)} = (\Delta A_{DPPH} - \Delta A_{sample} - \Delta A_{corr}) / \Delta A_{DPPH} \times 100.^{‡} \quad (1)$$

Ascorbic acid was used as a positive control. In parallel with the samples, spectra of the DPPH probe were also collected. All calculations include the ΔA_{DPPH} values taken at the same time as the corresponding values of the samples. ΔA_{corr} is a difference in absorbances of the blank PS-C_{60} solution recorded on the same wavelengths and with the same concentration of the PS-C_{60} as the corresponding sample-DPPH mixture. IC_{50} values were determined graphically as a concentration of the sample that consumed 50% of DPPH after period of 120 minutes (Fig. 6, SM1: Fig. S5, Table S2).

2.6. β-Carotene bleaching assay

The β-carotene/linoleic acid emulsion was prepared as follows: chloroform solutions of linoleic acid (40 mg), Tween 40 (400 mg) and β-carotene (0.6 mg) were mixed in round-bottomed flask and the solvent was removed *in vacuo.* The obtained residue was dispersed immediately in oxygenated HPLC grade water (47.5 mL) by vigorous shaking.

Oxidation reactions were carried out in 20 mL vials with screw caps at 50 °C. Freshly prepared β-carotene/ linoleic acid emulsion (4.75 mL) was stirred with the sample (0.25 mL) and process was monitored by measuring the decrease of β-carotene absorbance. Aliquots (0.33 mL) were taken at specific time intervals (0, 15, 30, 45, 60, 90,120,150, 180, 240, 300 minutes), diluted with ethanol (1:2, V/V) and absorbance at 470 nm was measured (SM1: Table S3, Fig. S6).

Samples of PS and PS-C_{60} hybrids were freshly prepared by stirring lyophilized material in deionized water at room temperature overnight. BHT solution (0.13 g/L, 0.6 mM) in methanol was used as a positive control. The negative control contained methanol instead of sample.

Final concentrations of PS-C_{60} complexes were in the range of 0.01 mg/mL - 0.1 mg/mL, while concentrations of BHT were 0.3 μg/mL and 6.0 μg/mL.

Antioxidant activity (SM1: Table S4) was expressed as inhibition percentage in relation to the control and calculated according to Eq. (2) [44].

$$\text{AA (\%)} = (DR_C - DR_S)/DR_C \times 100 \quad (2)$$

where AA—antioxidant activity, DR_C—degradation rate of β-carotene in the control sample = {[ln (a/b)]/t}, DR_S—degradation rate of β-carotene in the antioxidant sample = {[ln (a/b)]/t}, a = absorbance at time = 0, b = absorbance at 180 min), t = time.

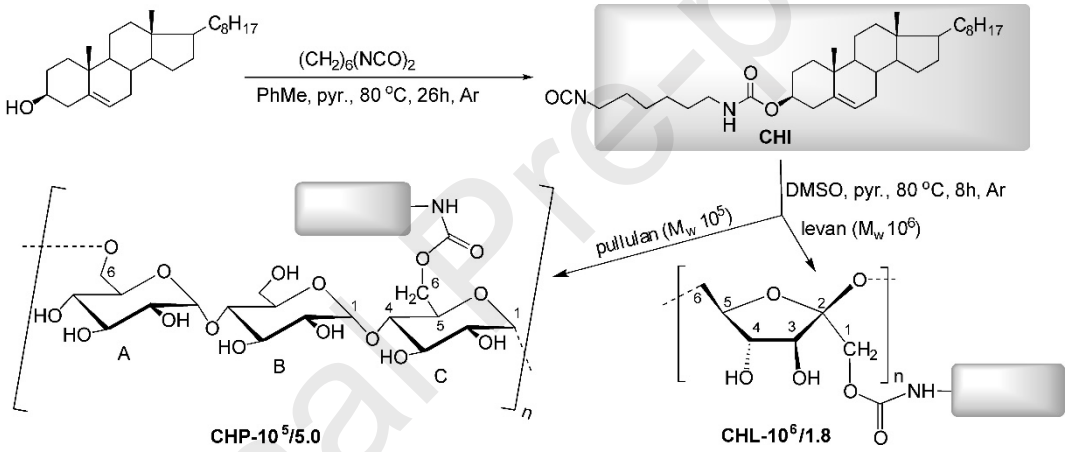
† All concentrations are expressed as final concentrations in the probe. Note that the initial concentrations of the aqueous solutions of the samples are three times higher before mixing with methanol and DPPH solution.

‡ $\Delta A = A_{max} - A_{min}$; ΔA_{corr} = absorbance difference of the blank PS-C_{60} probe at the same wavelengths as ΔA_{sample}.

3. Results and discussion

3.1. Synthesis and characterization of hydrophobized polysaccharides CHP and CHL

Following the reported procedure [33], target hydrophobically modified polysaccharides, CHP and CHL, were synthesized by a sequential alcoholysis reaction of isocyanate derivatives with cholesterol and native sugars, as shown in Scheme 1. In the first step, cholesteryl *N*-(6-isocyanatohexyl)carbamate, CHI, was prepared in a yield of 49% by reacting cholesterol with 1,6-hexyldiisocyanate in dry toluene and dry pyridine as catalyst at 80 °C for 26 h. In the second step, pullulan (M_w~10^5) and levan (M_w>10^6) have been modified using the steroid-isocyanate (CHI) alcoholysis in the DMSO/pyridine solvent mixture at 80 °C for 8 h. The modified products were purified from low-molecular-weight reagents and by-products by precipitation and dialysis, and then lyophilized. The partially hydrophobized pullulan (CHP) and levan (CHL) were obtained in moderate yields of 46% and 87%, respectively. The efficiency of a two-step purification procedure of CHP and CHL was verified by SEC analysis. The superimposed SEC chromatograms (pullulan/CHP and levan/CHL) are shown in Figure S1 (SM1). As can be observed from the shape of the curves in the HPLC determination, both native polysaccharides and their derivatives have a similar shape, with a slightly delayed retention time for the native polysaccharides. Also, the SEC chromatogram analysis of CHP and CHL revealed that there are no peaks corresponding to small molecules, confirming the absence of free steroid derivatives. The substitution degree of cholesterol residues in obtained polysaccharide derivatives, CHP and CHL, was 5.0 per 100 glucose units for pullulan (CHP-10^5/5.0) and 1.8 per 100 fructose units for levan (CHL-10^6/1.8) as measured by ^{1}H NMR (see Fig. 2A and NMR analysis).[§]



Scheme 1. Synthetic route towards cholesterol-modified pullulan (CHP) and levan (CHL).

The IR, 1D and 2D NMR spectra, and spectral data of starting compounds and modified polysaccharides are given in Experimental part and SM2. The detailed characterization of polysaccharide-cholesterol conjugates CHP and CHL was based on an extensive analysis of their IR (Fig. 1) and NMR spectra and confirmed by comparison with those of the parent polysaccharides and steroid derivative (Fig. 2B,C). Fig. 1 shows the IR spectra for cholesteryl *N*-(6-isocyanatohexyl)carbamate (CHI), unmodified polysaccharides, pullulan (P) and levan (L), and hydrophobized polysaccharides, CHP and CHL. Compared with cholesterol derivative (CHI) and both polysaccharides (P and L), the appearance of a new weak broad absorption band around 1690 cm^{-1} for the carbamate carbonyl stretching vibrations (next to the peak at 1640 cm^{-1} which assigned to O–H bending vibrations of water) along with the characteristic bands of pullulan and levan skeletons indicated that the free NCO group of CHI reacted with a polysaccharide hydroxyl groups.

[§] The degree of substitution (DS) values of the cholesterol units per 100 monosaccharide units in CHP and CHL was determined by the ratio of the integral values of the angular Me(18) group protons singlet at δ 0.65 ppm to sugar protons at C(6) position (H-$6^{P(C)}$) of glucose unit C (Glc(α1–6) at δ 3.88 ppm for CHP) and protons singlet at C(3) (H-3^{L} at δ 3.98 ppm for CHL) in their ^{1}H NMR spectra (Fig. 2A).

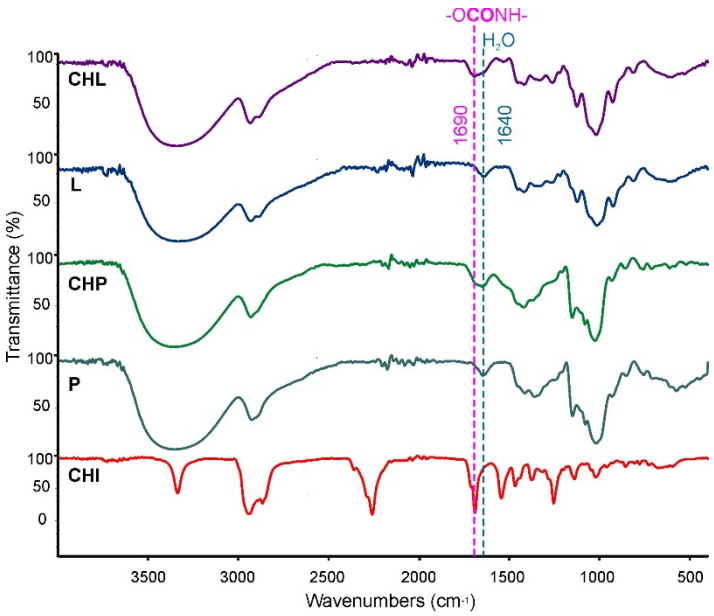


Fig. 1. Comparison of the IR spectra of cholesteryl *N*-(6-isocyanatohexyl)carbamate (CHI), pullulan (P), CHP, levan (L), and CHL.

Fig. 2B,C compares the ^{1}H and ^{13}C NMR spectra of steroid derivative (CHI), pullulan (P) and levan (L) with the spectra of CHP and CHL. In addition to the polysaccharide characteristic signals in the 3-6 ppm range ^{1}H NMR spectra of CHP and CHL contain the overlapped signals belonging to the steroid unit (H-3 at δ 4.29 ppm; H-6 at δ 5.33 ppm) in the same range (SM2: HSQC of CHL (p. 33) and CHP (pp. 19-21)) and isolated signals in the upfield region (δ 0.5-2.5 ppm) with characteristic shifts of angular methyl groups (C(18) and C(19) singlets at δ 0.65 and 0.96 ppm, respectively), as well as two doublets of C(21)- and C(26,27)-methyl groups at δ 0.89 and 0.84 ppm, respectively. It is important to note that the peak of angular 18-Me group at δ 0.65 ppm as a separated signal was used for the degree of substitution (DS) determination. Further confirmation of the presence of cholesterol moiety in CHP and CHL conjugates was the appearance of low intensity carbon resonances in the 10-60 ppm range of their ^{13}C NMR spectra. Also, next to the signals belonging to unmodified polysaccharides the two signals of steroid unit at δ 122 (C-6) and 73 ppm (C-3) in the ^{13}C NMR spectra of CHP and CHL (present in their HSQC spectra, SM2: pp. 19-21, 33) corroborated the presence of the covalently incorporated cholesterol moiety.

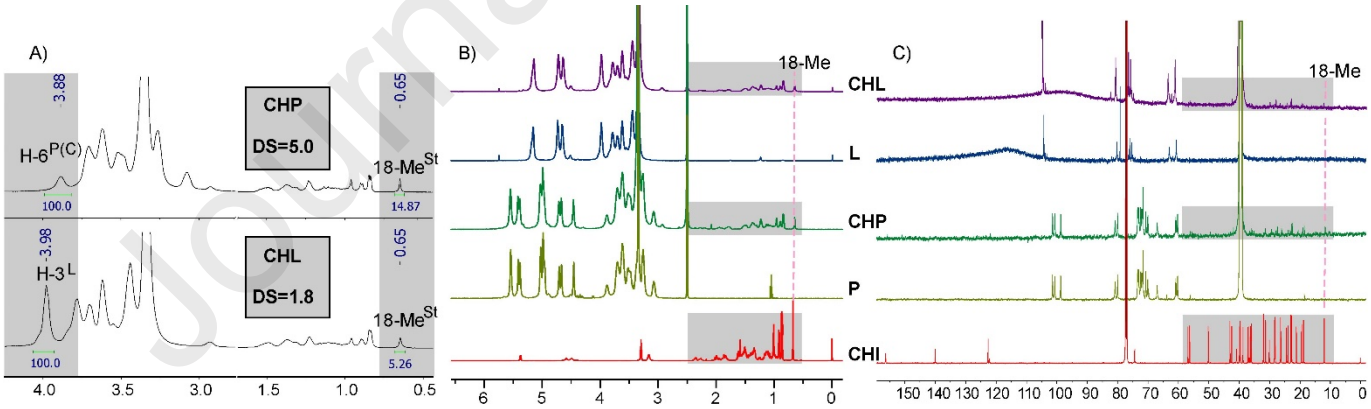


Fig. 2. A) The degree of substitution (DS) of hydrophobized polysaccharides CHP and CHL; B) ^{1}H NMR; and C) ^{13}C NMR spectra of cholesteryl *N*-(6-isocyanatohexyl)carbamate (CHI), pullulan (P), CHP, levan (L), and CHL.

3.2. Preparation and characterization of L-C_{60}, CHL-C_{60} and CHP-C_{60} supramolecular hybrids

PS-C_{60} supramolecular hybrids were obtained by a three-step strategy based on mixing of separately prepared solutions of components in corresponding solvents. In the first step, the polysaccharide was suspended in distilled water with initial concentration of 0.05 mg/mL and stirred vigorously at 55 °C for 72 h. The solution of C_{60} in pyridine (c=0.5 mg/mL) was added in the aqueous solution of PS (C_{60}:PS weight ratio: 1:2.4) (the initial concentration of C_{60} in a water/pyridine system was 0.02 mg/mL). The amount of fullerene added was 29.4 wt % with respect to the PS-C_{60} mixture (Table 1). After mixing the two components, the resulting yellow solution was stirred in the dark for 72 h at

room temperature. In the second step, the solution was dialyzed against distilled water to remove pyridine and then filtered through 0.45 μm PTFE syringe filter (although there were no visible particles). The resulting clear yellow solutions** were analyzed (C_{60} content determination, DLS, SEM), and finally, freeze-dried to dark brown solids in the moderate to good yields (L-C_{60}, 78%, CHL-C_{60} 82%, and CHP-C_{60} 40%). Lyophilized samples of the obtained noncovalent hybrids are stored in the dark at room temperature and used for characterization and further studies. The concentration of the solubilized C_{60} in dialyzed PS-C_{60} samples, spectroscopically determined in toluene extracts, was in the range of 6.8-9.2 μg/mL, meaning that effectiveness of solubilization was in the range of 31-46% (45.9% for L-C_{60}, 40.1% for CHL-C_{60} and 30.7% for CHP-C_{60}) (SM1: Table S1). UV-Vis spectra of the toluene solution of C_{60} extracted from the obtained dialyzed samples are shown in Fig. 3B. Also, the initial C_{60} loading and C_{60} weight percentages in solid PS-C_{60} formulations are presented in Table 1.

Table 1
Concentrations of solubilized C_{60} for PS-C_{60} in water, the initial C_{60} loading and weight percentages of C_{60} in freeze-dried PS-C_{60} powders.

Sample Name	C_{60} concentration[a]		C_{60} w/w % in PS-C_{60} [b]	Initial C_{60} loading [c]	
	w/V (g/L)	(mol/L)		w/V (g/L)	w/w %
L-C_{60}	9.18×10^{-3}	1.27×10^{-5}	18.0	2.00×10^{-2}	29.4
CHL-C_{60}	8.42×10^{-3}	1.17×10^{-5}	19.3	2.00×10^{-2}	29.4
CHP-C_{60}	6.76×10^{-3}	9.38×10^{-6}	24.7	2.00×10^{-2}	29.4

[a] Concentrations of C_{60} in PS-C_{60} after dialysis determined from toluene extracts of retentates (for details see Table S1).
[b] C_{60} w/w % = 100 × (c(C_{60}) × V / m(PS-C_{60})); V- volume of solution subjected to lyophilization; m - PS-C_{60} powder.
[c] Initial concentration and percentage of C_{60} per total weight of PS-C_{60} mixture.

The formation of L-C_{60}, CHL-C_{60} and CHP-C_{60} was confirmed by UV-Vis and FTIR spectra, and by comparison with those of the parent polysaccharides and C_{60}. The UV-Vis spectra of stable yellow dialyzed fullerene-bearing carbohydrate materials and parent C_{60},†† were measured in the 300–800 nm range. As shown in Fig. 3A, in that range of wavelengths all three spectra are almost identical to that of C_{60} in water and consist two characteristic fullerene absorption bands, one strong at about 340 nm and the shoulder at about 450 nm, attributed to the formation of PS-C_{60} containing aqueous systems. The absorption at 342.5 nm which is characteristic for C_{60}/water system is slightly shifted for hybrids to 340.0 (L-C_{60}), 339.0 (CHL-C_{60}) and 340.5 nm (CHP-C_{60}). Also, the wavelength values for all four samples are red-shifted in comparison with those of free C_{60} in toluene (333 nm), confirming that C_{60} molecules were solubilized in water in the presence of PS (Fig. 3B) [45].

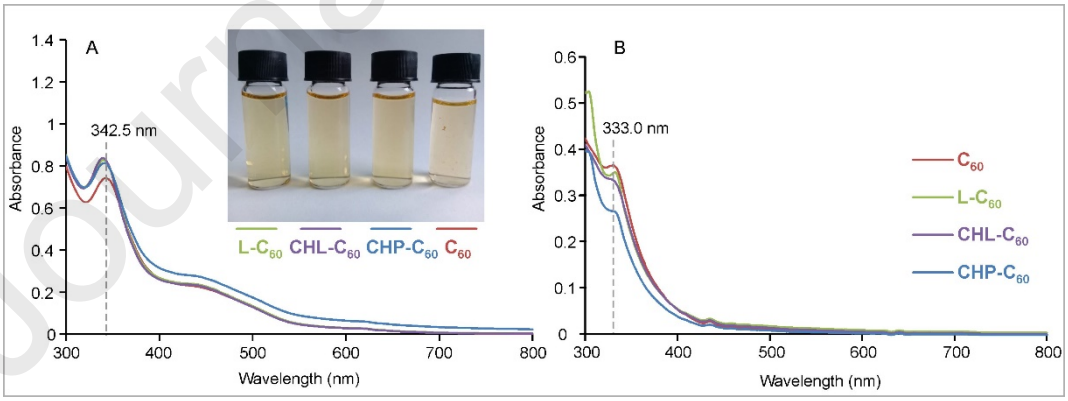


Fig. 3. Comparison of UV-vis spectra of L-C_{60}, CHL-C_{60}, CHP-C_{60}, and pristine C_{60} in water, prepared under identical conditions (A) and those of their toluene extracts (B).

** These solutions are stable without visible particles or precipitate for at least four months after preparation. In addition, the UV-Vis spectra performed on the same samples during four months remain almost unchanged in terms of shape and intensity for all three supramolecular hybrids, but not for C_{60} alone, indicating their stability (SM1: Fig. S2).

†† Colloidal C_{60} particles in water were prepared in the same way as the PS-C_{60} hybrid materials. Unlike PS-C_{60} retentates, they showed much lower stability, since immediately after filtration of the resulting retentate, fullerene precipitation was observed.

The IR spectra of the solid PS-C_{60} samples together with the reference spectrum of pristine C_{60} fullerene powder are shown in Fig. 4. The spectrum of C_{60} displays four vibrational IR absorption lines at 1426, 1180, 573, and 522 cm^{-1}. The spectra of PS-C_{60} showed that polysaccharide absorptions overlap fullerene bands at 1426 and 1180 cm^{-1}. Only two stronger bands (524.1and 576.3 cm^{-1} for L-C_{60}; 522.3 and 572.9 cm^{-1} for CHL-C_{60}; 524.7 and 577.0 cm^{-1} for CHP-C_{60}), visible in their IR spectra, can be unequivocally ascribed to C_{60}.

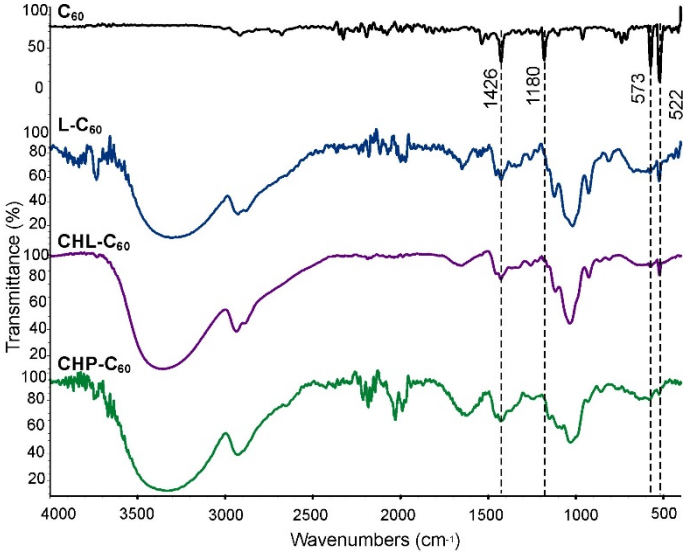


Fig. 4. Comparison of the IR spectra of C_{60}, L-C_{60}, CHL-C_{60} and CHP-C_{60}.

The self-assembling and the particle size distributions (PSD) of the obtained dialyzed PS-C_{60} solutions were studied by SEM and DLS. In order to examine the influence of the fullerene moiety on the particle size and shape, the aggregation behavior of the parent polysaccharides was also included in this investigation. The three pairs of analyzed samples (PS/PS-C_{60}) had acceptable polydispersity index (PdI) values from 0.112 to 0.299, indicating a moderate polydispersity. A clearly monomodal PSD was obtained for L and L-C_{60}, while more or less pronounced bimodal distribution could be assigned for the other studied samples. The presence of a small amount of aggregates in the micrometric range was visible for CHL/CHL-C_{60} pair, while two distinct size populations appeared in roughly nanometric domain for the CHP/CHP-C_{60} pair (Fig. 5, Table 2). However, the most important feature in all presented PSDs is a shift in particles size towards smaller diameter values upon incorporation of C_{60} into the PS unit. As presented in Table 2, the Z-average hydrodynamic diameters (Z_{av}) of the corresponding pairs of PS/PS-C_{60} nanoparticles (L/L-C_{60}, CHL/CHL-C_{60}, and CHP/CHP-C_{60}) were 138.00/80.84, 136.50/51.95, and 189.80/64.90 nm, respectively. Supramolecular incorporation of C_{60} into the studied carbohydrates reduced the size of the nanoparticles in all three cases, with a greater difference in the case of C_{60}-bearing hydrophobized polysaccharides. The degree of cholesterol substitution in PS has direct influence on the C_{60}-content in the corresponding PS-C_{60} complex (L-C_{60} (18%), CHL-C_{60} (19%), CHP-C_{60} (25%)), Table 1). It should be pointed out that with increasing overall hydrophobicity of PS-C_{60} nanoparticles and C_{60} content in them the reduction factor of the *Z*-average diameters grows (Levan/L-C_{60} 1.7, CHL/CHL-C_{60} 2.6, CHP/CHP-C_{60} 2.9), indicating the crucial role of the C_{60} content for these differences. The PDIs decreased slightly in the same manner. The presented results explain greater stability of the C_{60}-containing carbohydrate nanoparticles in water compared to the corresponding native polysaccharide nanoparticles, due to hydrophobic interactions of the steroid and the fullerene units, which enable the cohesion of the starting nanoparticles.

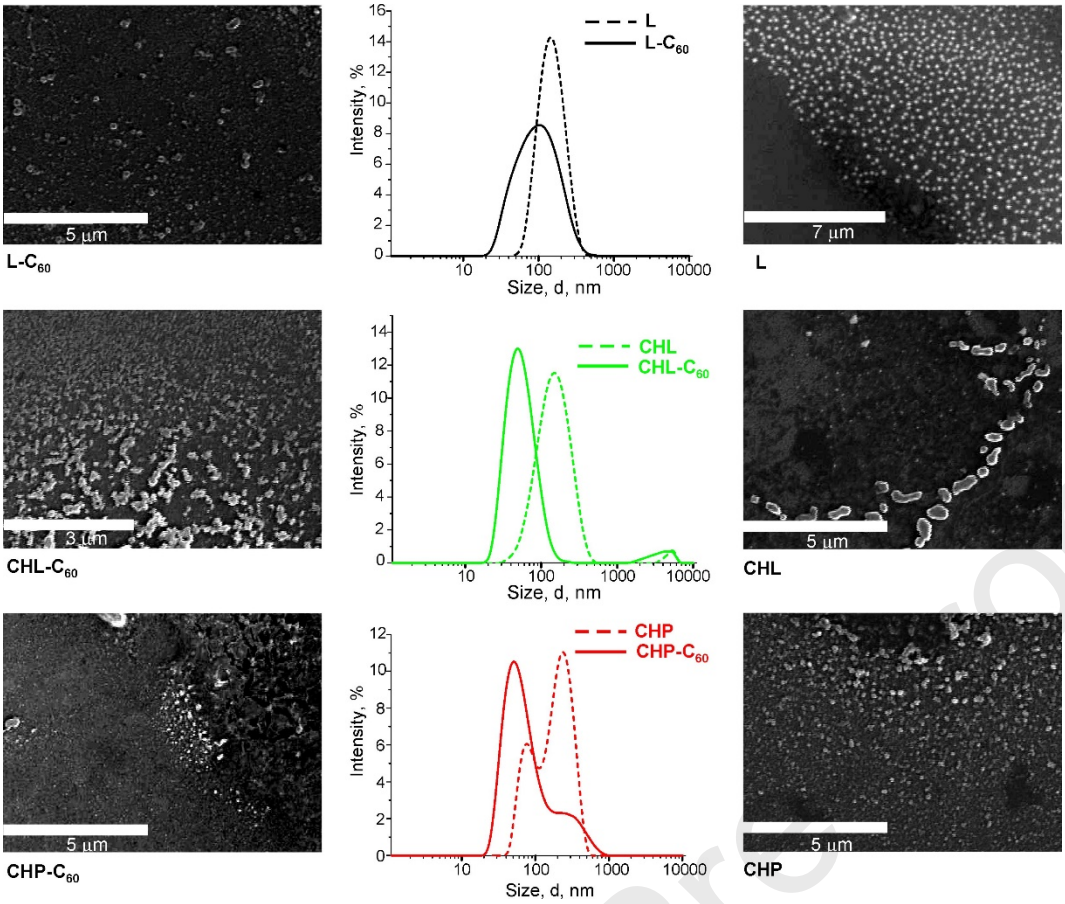


Fig. 5. Comparison of intensity-weighted PSD for aqueous suspensions of the parent polysaccharides (L, CHL and CHP, dashed line) and PS-C_{60} retentates (full line), obtained from DLS and typical SEM images of their self-organized nanoparticles after evaporation of aqueous solution deposited on a glass substrate at room temperature.

Table 2
DLS (Z-average hydrodynamic diameter (Z_{av}), polydispersity index (PDI), PSD by intensity: mean size of fractions (peaks), and their relative ratio (%)) and SEM data (mean particle size) for parent PS and PS-C_{60}.

	DLS						SEM
Sample Name			**Peak 1**		**Peak 2**		**Mean particle size, d, nm**
	Z_{av}, nm	**PdI**	**Size, d, nm**	**Intensity, %**	**Size, d, nm**	**Intensity, %**	
L	138.00	0.112	157.40	100	-	-	112.81
L-C_{60}	80.84	0.237	111.20	100	-	-	82.77
CHL	136.50	0.224	158.40	98.2	5017	1.8	132.84
CHL-C_{60}	51.95	0.206	57.87	95.8	3819	4.2	48.36
CHP	189.80	0.299	238.00	68.3	84.77	31.7	130.91
CHP-C_{60}	64.90	0.261	69.74	84.1	332.0	15.9	63.60

The morphology of PS-C_{60} hybrid nanoparticles was studied by SEM on the samples prepared by a drop-drying process [46]. Representative SEM images of the same samples which were used for DLS study, obtained by slow evaporation of aqueous solution on a glass substrate at room temperature, are given in Fig. 5. Depending on the nature of the polysaccharides, the self-organized morphologies vary slightly from dominant individual rounded particles to elongated structures that tend to aggregate into network structures, especially pronounced in the case of CHL and the CHL-C_{60} hybrid nanoparticles. The particle size analysis was performed using the image analysis software (Infinity Analyze 6.2.) by measuring the diameter of particles from SEM images. Histograms of particles representing the particle diameter distribution and parameters which describe particles are presented in Fig. S7 (SM1). Comparison of the particle size distributions measured by SEM with those from DLS data revealed the same trend in the decreasing of the particle size of PS-C_{60} compared to the PS itself (112.81/82.77, 132.84/48.36 130.91/63.60 for L/L-C_{60}, CHL/CHL-C_{60}, and CHP/CHP-C_{60}, respectively) (Table 2, Fig. 5.).

3.3. Antioxidant activity

We have investigated *in vitro* antioxidant activity of aqueous solutions of PS-C_{60} hybride materials and parent polysaccharides by means of DPPH radical scavenging and β-carotene-linoleic acid bleaching assays with different mechanism of antioxidant action.

3.3.1. DPPH radical scavenging activity

As already mentioned, fullerene C_{60} has a high affinity for free radical species (commonly is described as a „radical sponge“) [40]. The DPPH radical assay, as a simple method through which we gain insight into the radical scavenging abillity, is often used for preliminary examination of antioxidant activity of C_{60} and its derivatives [25,28,47,48]. In this work, aqueous solutions of PS-C_{60} hybride materials were prepared, and their activity against DPPH radical was examined. Ascorbic acid was used as a positive control.
The free radical scavenging activity (RSA) of prepared PS-C_{60} supramolecular hybrids was measured according to procedure developed by Mensor at al., with some modifications [49-52]. DPPH radical scavenging of PS-C_{60} sample solutions at five different concentrations (four same concentrations for all three samples: 0.333, 0.167, 0.084, 0.042 mg/mL, as well as the lowest concentrations of 0.017, 0.018, and 0.0056 mg/mL for L/C_{60}, CHL/C_{60} and CHP/C_{60} dialyzed samples, respectively), was monitored by UV-Vis spectroscopy (SM1: Figs S3-S5 and Table S2). Stronger attenuation of absorbance at 521 nm means higher activity. More concentrated samples reacted with almost total depletion of DPPH within the first few minutes and change of colour from purple to pale orange. Samples at concentrations lower than 0.167 mg/mL only brightened during the observation, indicating uncomplete consumption of DPPH (SM1: Fig. S4). The scattering effect resulting from the presence of suspended matter (detectable as vertical shift of spectra), as well as the overlapping of the intrinsic absorption of the C_{60}, resulted in the use of Eq. (1) instead of the usual formula containing the directly measured absorbances at 521 nm. All three PS-C_{60} reacted with DPPH in a concentration-depended manner, with IC_{50} values‡‡ of 0.076 mg/mL, 0.104 mg/mL and 0.073 mg/mL for L-C_{60}, CHL-C_{60} and CHP-C_{60}, respectively (Fig. 6; SM1: Figs S4 and S5 and Table S2).

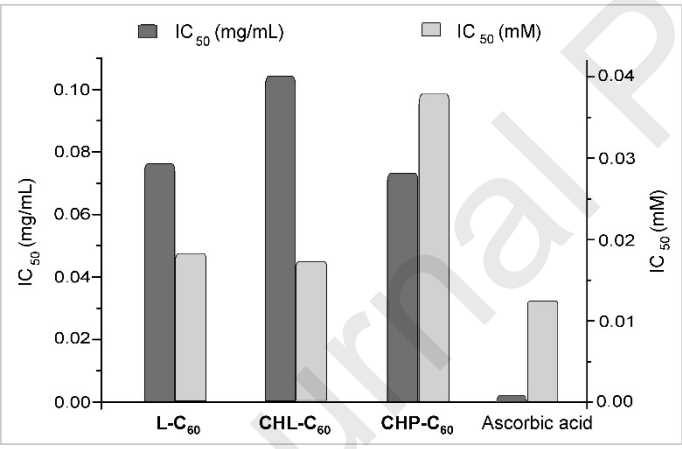


Fig. 6. IC_{50} values of the PS-C_{60} nanoparticles and control ascorbic acid, expressed as a concentration (in mg/mL of sample-dark blue or mM of active component-light blue) that consumes 50% of DPPH radical, after 2 h.

Control experiments with the different concentrations of the parent polysaccharides showed no significant activity for all samples, except for the highest concentration of levan (0.333 mg/mL; RSA=20.1 % DPPH) (SM1: Table S2). Positive control, ascorbic acid, at the same conditions, showed much lower IC_{50} value than the samples (IC_{50}=0.0022 mg/mL). However, IC_{50} values of the samples expressed as molar concentrations of the contained C_{60}, are much closer to the one of the ascorbic acid (0.018 mM, 0.017 mM and 0.038 mM (of C_{60}) for L-C_{60}, CHL-C_{60} and CHP-C_{60}, *vs* 0.0125 mM for ascorbic acid, Fig. 6). Although we expected that the radical scavenging activity of the PS-C_{60} nanoparticles will depend solely on the content of fullerene, as an active component of the hybrid material, differences among molar IC_{50} values indicated their dependence on the type of polysaccharide subunit. It could be supposed that observed dependence resulted from a different shielding effect of the polysaccharides on the fullerene core inside the complexes, which is closely related to the level of hydrophobization of the polysaccharide components. The mild levan's activity also contributes to the activity of levan-C_{60}, compared to the CHL-C_{60} and CHP-C_{60}.

‡‡ IC_{50} value is expressed as a sample concentration that consumes 50% of the initial DPPH radicals after 2 hours.

3.3.2. β-Carotene-linoleic acid bleaching inhibitory activity

The β-carotene bleaching assay is an *in vitro* assay often used to study antioxidant activity of C_{60} derivatives and its complexes [53-55]. The method is based on the principle that linoleic acid oxidized products initiate degradation of highly conjugated β-carotene resulting in discoloration of emulsion. Antioxidants reduce the extent of discoloration by consumption of linoleic acid – free radicals and other oxidation products formed. As a positive reference, BHT was used.

In the present study slightly modified method reported by Marco was used [56]. Three concentrations of PS-C_{60} (0.01, 0.05 and 0.1 mg/mL), parent PS (0.1 mg/mL) along with the standard antioxidant BHT at two levels (0.3, 6 μg/mL) were tested. The reaction was monitored by the decrease in absorbance at 470 nm during 300 min (SM1: Table S3, Fig. S6) and the calculation of antioxidant activity was performed in relation to the absorbance of the sample at zero time. It is noticeable that the initial absorbance of the tested emulsions is in the range of 0.7-1, which is the result of the color of PS-C_{60} complexes and the presence of polysaccharides (SM1: Table S3). Fig. S6 (SM1) clearly shows that the absorbance curves of PS-C_{60} containing probes at the highest concentration (0.1 mg/mL) are close to those of BHT standard (6 μg/mL), while absorbance changes of the emulsions with parent polysaccharides are similar to the control. Antioxidant activity (AA) was calculated according to Eq. (2) [44], and expressed as an inhibition percentage at 180 min in relation to control (Fig. 7, SM1: Table S4). It is observed that the CHP- C_{60} and CHL-C_{60} complexes at the highest applied concentration showed AA higher than 50 %, while the activity of L-C_{60} complex was somewhat lower. For all C_{60}-containing nanoparticles, the activity varied in a concentration-dependent manner. At the same time, samples of parent polysaccharides exhibited negligible activity (<10%). Although the CHP-C_{60} supramolecular hybrid is the most potent, it is evident that the hydrophobization of levan has a positive effect on the expression of antioxidant activity in this assay system. Differences in the antioxidant activity of L-C_{60} and CHL-C_{60} complexes in DPPH and β-carotene/linoleic acid systems indicate that the solubility of the polysaccharide used as the carrier plays a significant role.

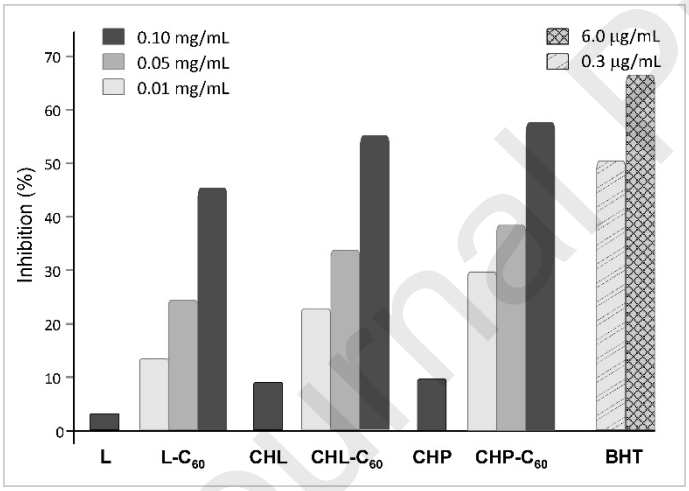


Fig. 7. Antioxidant activity of the PS-C_{60} nanoparticles and positive control BHT in β-carotene/linoleic acid system, expressed as the percent of inhibition at 180 minutes relative to control sample. PS-C_{60} nanoparticles were applied in three different concentrations (0.1 mg/mL; 0.05 mg/mL and 0.01 mg/mL), meaning that applied molar concentrations of active component C_{60} are as listed: 0.024 mM for L-C_{60}; 0.016 mM for CHL-C_{60} and 0.052 mM for CHP-C_{60} for the most concentrated samples and their 2-fold and 10-fold dilutions; BHT concentrations are 0.027 mM and 0.0014 mM.

4. Conclusions

In summary, three polysaccharide-C_{60} supramolecular hybrids were synthesized, characterized, and their antioxidant activities evaluated. Hydrophobized polysaccharides, CHL and CHP, with a degree of substitution of 1.8 for levan and 5.0 for pullulan were obtained using the alcoholysis reaction of cholesteryl isocyanate derivative under the same conditions. Polysaccharides levan, cholesterol-hydrophobized levan and the corresponding analog of pullulan, which is a known compound, were selected as carriers of the fullerene C_{60} in order to improve its solubility and stability in water, and to study its antioxidant properties. The formation of supramolecular hybrids has been studied and confirmed by IR and UV-Vis spectroscopies. In addition, we have studied their particle size distributions and morphology in aqueous solution, using DLS and SEM. The size of self-assembled C_{60}-carbohydrate nanoparticles in water was reduced

compared to the corresponding starting polysaccharide nanoparticles due to hydrophobic interactions of the fullerene with steroid units and/or hydrocarbon skeleton of the polysaccharide, which allow the building of more compact nanostructures. The DLS results were in line with the SEM measurements. According to the SEM micrographs, the morphology of the nanoparticles can be considered as spherical basic structures, which have strong capability of networking in the case of levan-containing nanoparticles. We present DPPH free radical scavenger and β-carotene bleaching abilities of the synthesized noncovalent hybrids compared to native polysaccharides, as well as ascorbic acid and BHT as positive controls, respectively. The antioxidant potential of the analyzed samples in both performed assays was concentration dependent. Among the tested supramolecular complexes, CHP-C_{60} was found to be the most efficient DPPH radical scavenger and the best inhibitor of β-carotene bleaching assay. In general, the present study clearly demonstrated that PS-C_{60} supramolecular hybrids displayed moderately antioxidant properties in both assays, with the increasing of the activity relative to the growing hydrophobicity. The above results suggest that the antioxidant activity of the PS-C_{60} nanoparticles could be attributed to the combined effects of the fullerene content, the degree of hydrophobization and the shielding effect of polysacharide units on the C_{60} core. In short, we present a way to solubilize and stabilize fullerene to make it applicable for biological studies.

Acknowledgments

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Data availability statement

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Appendix:

Supplementary material 1: Tables S1-S4 and Figs S1−S7.

Supplementary material 2: IR and NMR spectra of CHI, pullulan, levan, CHP and CHL, IR spectra of C_{60} and PS-C_{60} complexes.

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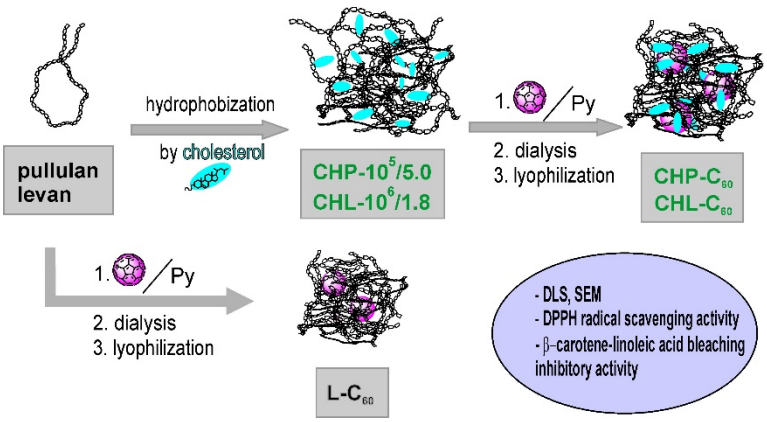
Graphical abstract for TOC:

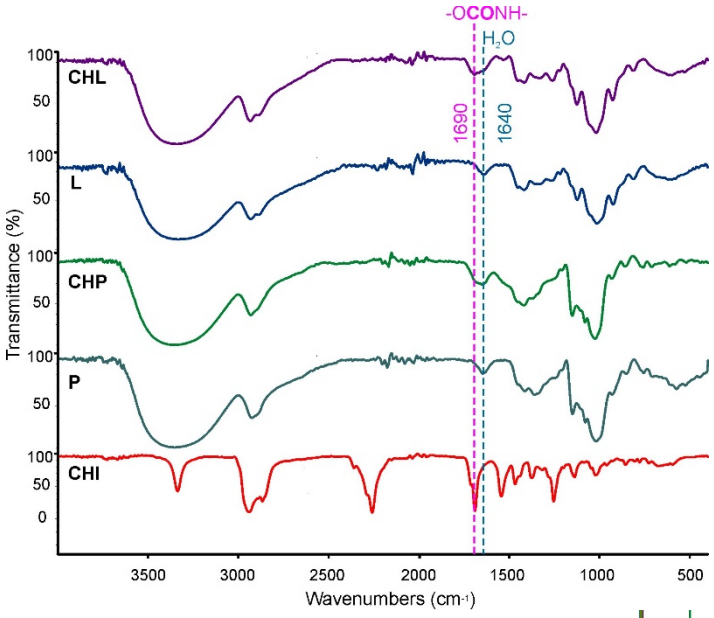
Polysaccharide-fullerene supramolecular hybrids: synthesis, characterization and antioxidant activity

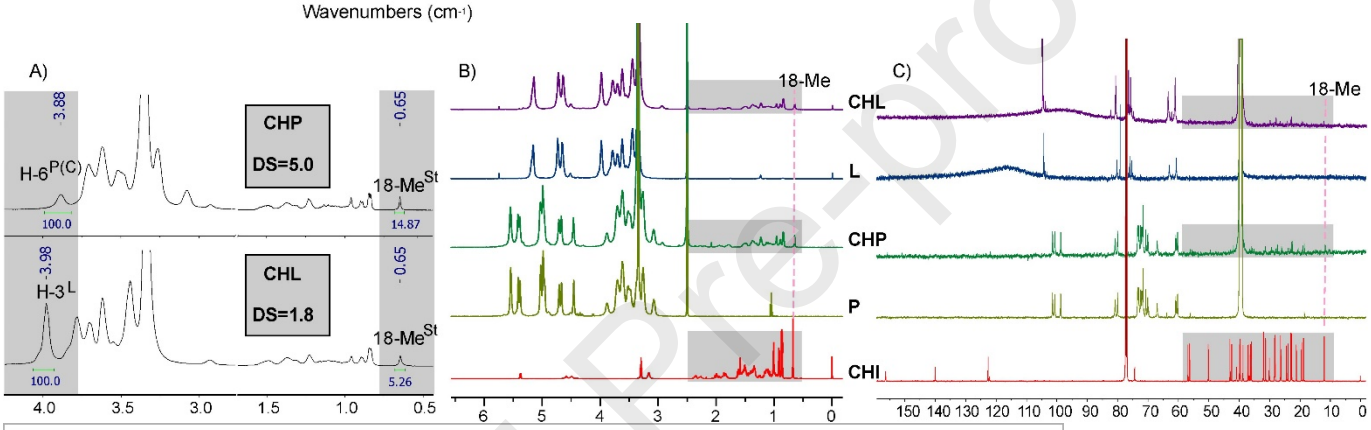
Tatjana J. Kop, Dragica M. Jakovljević, Ljiljana S. Živković, Andrijana Žekić, Vladimir P. Beškoski, Dragana R. Milić, Gordana D. Gojgić-Cvijović, Mira S. Bjelaković*

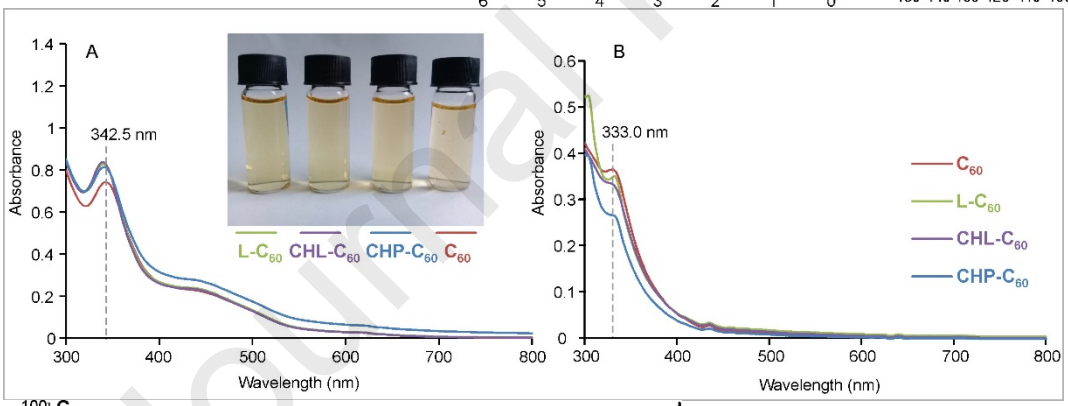


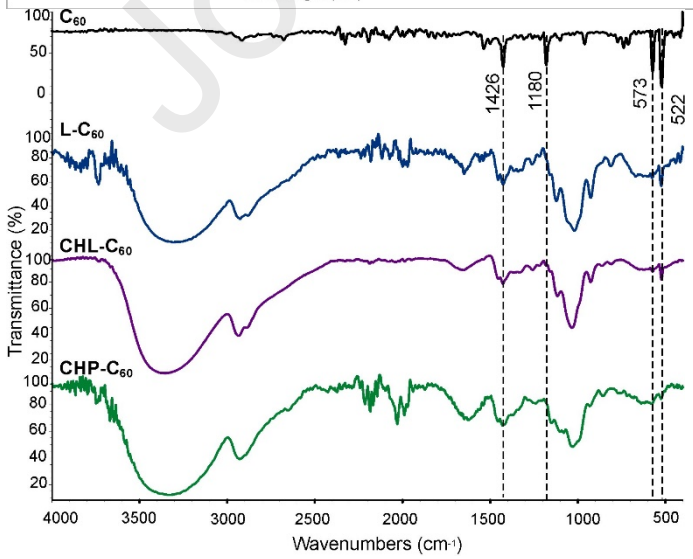
Highlights

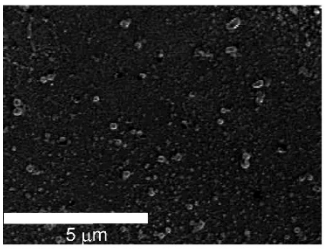
1. Polysaccharides (PS) levan and pullulan were hydrophobized by cholesterol.
2. New C_{60}-PS non-covalent hybrid NPs were synthesized and studied by DLS and SEM.
3. The size of C_{60}-PS NPs in water was reduced compared to starting PS NPs.
4. DPPH radical scavenging and β-carotene bleaching abilities of NPs were studied.
5. The antioxidant potency increases with the growing hydrophobicity.











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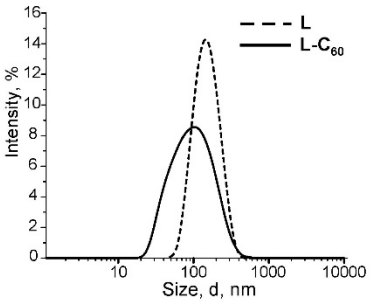

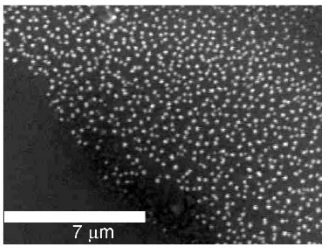

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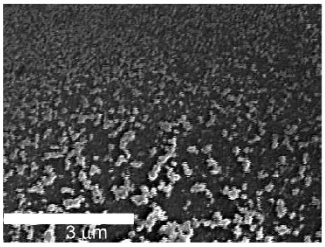
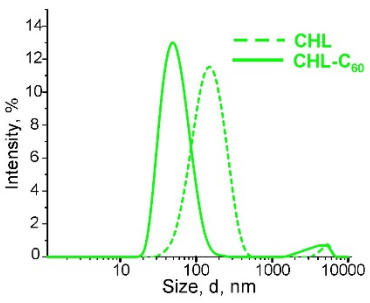
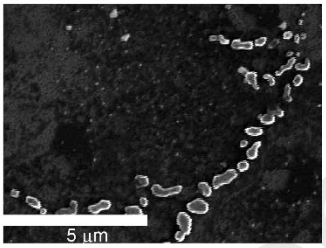
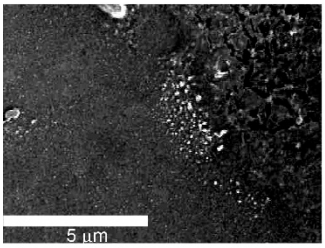


CHL-C_{60}

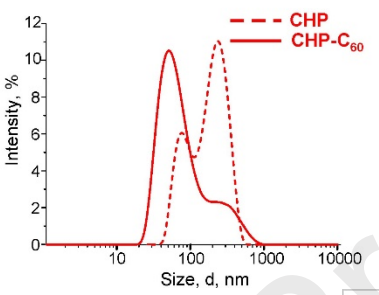


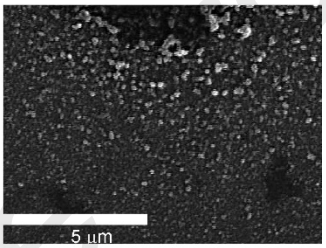


CHL

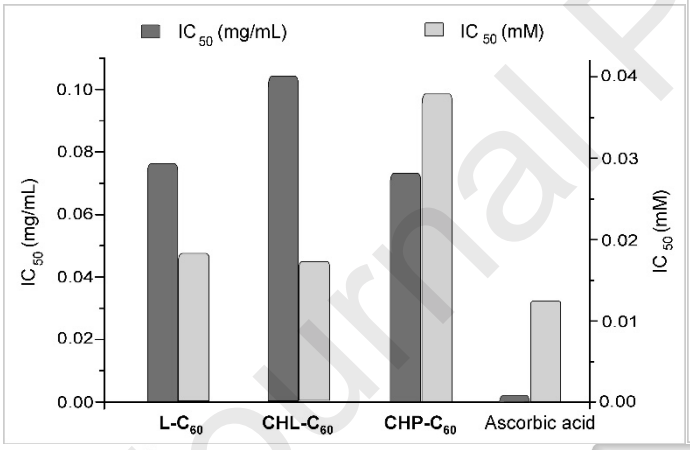


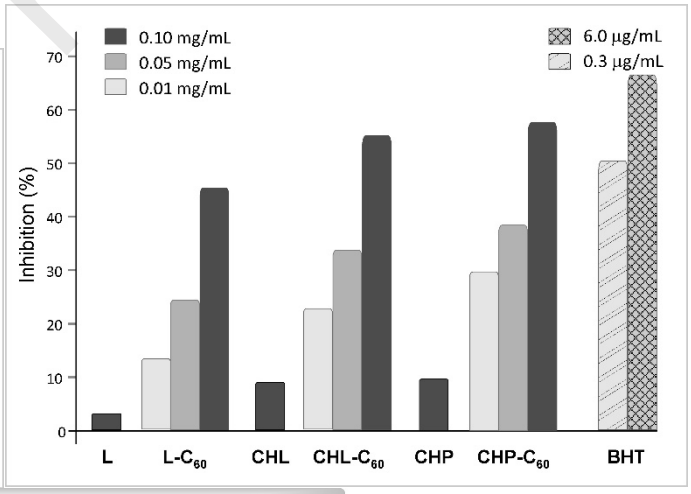
CHP-C_{60}

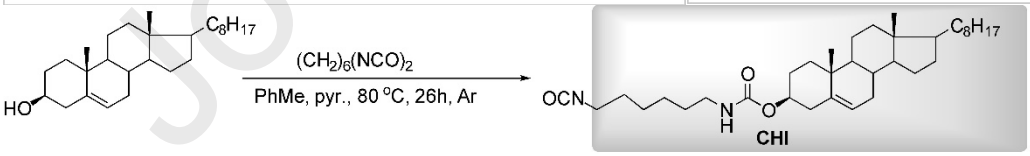


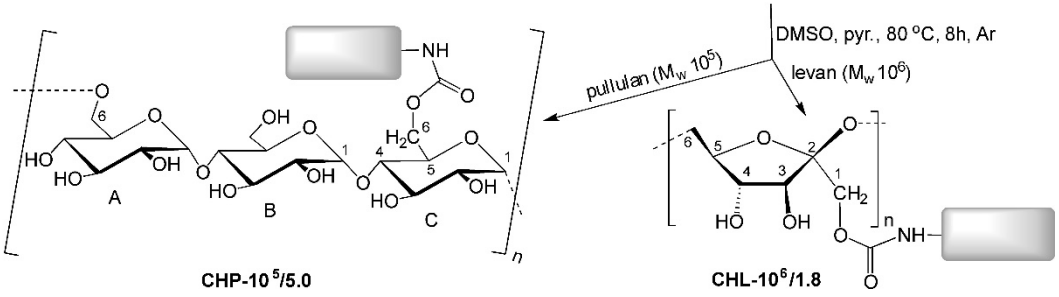


CHP









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Dragana R. Milić - Review

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Mira S. Bjelaković - Conceptualization; Investigation; Visualization; Writing

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: