New water-soluble forms of α-tocopherol: preparation and study of antioxidant activity *in vitro*

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Experimental

Materials

N-vinylpyrrolidone (99%, VP, Alfa Aesar) was distilled in vacuum to remove the NaOH inhibitor. Triethylene glycol dimethacrylate (95%, TEGDM, Aldrich) was used without additional purification. 2,2'-azo-bis-isobutyronitrile (AIBN) purified by recrystallization from ethanol was used as the initiator of radical polymerization. We used toluene (analytical grade) and *n*-hexane (reagent grade).

Synthesis of the copolymer

The synthesis of the copolymer was carried out in the three-necked flask equipped with the reflux condenser and the thermometer under continuous bubbling with argon for 2 hours at 80 °C in the thermostat. All components of the reaction mixture were introduced simultaneously. The content of reagents in the solvent was ~20 wt%. The concentration of the initiator in the solution is 0.02 mol L^{-1}. After completion of the reaction, toluene-soluble products were obtained; the reaction mixture was transparent and homogeneous.

The isolation of the copolymer from the toluene solution was carried out by precipitation with the tenfold excess of the precipitant, *n*-hexane. The precipitate was filtered on the Buchner funnel and dried from solvent / precipitant to constant weight in air and in vacuum. The copolymer yield was 94.5%.

Encapsulation of tocopherol in polymer particles

We used *D-α*-tocopherol (Alfa Aesar, TP):

Encapsulation into polymer particles was carried out using solutions of copolymer and tocopherol in isopropyl alcohol (Tables S1-S3). For this, different volumes of tocopherol solution were added dropwise to the copolymer solution under constant stirring with a magnetic stirrer. After drying from isopropyl alcohol, the polymer films containing tocopherol were dissolved in 8 ml of the aqueous neutral buffer solution (pH 6.8-7.0 or 7.2-7.4). Composition 0.01 M of the phosphate-salt buffer solution with pH 6.8-7.0: 137 mM NaCl, 2.7 mM KCl, 4.29 mM Na_2HPO_4, 1.47 mM KH_2PO_4. Composition 0.01 M of the phosphate-salt buffer solution with pH 7.2-7.4: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4.

Methods

Elemental analysis

Elemental analysis was used to determine the content of *CHN* atoms in the VP-TEGDM copolymer using the Vario cube Elementar GmbH device. Their content was 61.0, 8.9 and 11.2%, respectively. From the data on the nitrogen content, the content of VP units in the copolymer was calculated, which was 4.7 mol%.

Dynamic light scattering

The dynamic light scattering method was used to determine the hydrodynamic radii of the copolymer and the critical concentration of aggregation (CAC) in water. The hydrodynamic radius of scattering centers (R_h) for spherical particles is calculated using the Einstein-Stokes equation

$$D = k \times T \Big/ 6 \times \pi \times \eta \times R \,,$$

where D is the diffusion coefficient, k is the Boltzmann constant, T is the absolute temperature, and η is the viscosity of the medium, in which the dispersed particles are suspended.

The study of light scattering by aqueous solutions of copolymers was carried out on the detection angle of 90° by the Photocor Compact (Photocor LTD, Russia) at the wavelength of 654 nm. The solutions of the copolymer were previously passed through the filter with the pores of 0.45 μm in diameter. Before measuring, the vial with the solution was thermostated for *ca.* 30 min. The experimental data were processed using the DynaLS v. 2.8.3 software.

Determination of the absolute weight average molecular weight and the second virial coefficient of the VP-TEGDM copolymer

The absolute molecular weight of the copolymer in water was determined using the Debye method at $T = 22$ °C.[S1] For this, aqueous solutions of copolymers of specified concentrations were prepared and their light scattering intensity was measured. The refractive indices of copolymer solutions were measured with the refractometer. Then the increment of the refractive index dn/dc was determined from the concentration dependence of the refractive index of the solution. Based on the data obtained, the graph of the dependence of $K \times c / R_\Theta$ on c was constructed, which is a straight line. Extrapolating the resulting graph to the ordinate axis, a segment was obtained, the numerical value of which is $1/M_w$. The second virial coefficient was calculated using the formula $A_2 = b/2$, where b is the tangent of the angle of inclination of the straight line to the abscissa axis. The found A_2 value was $1.2 \times 10^{-4} \pm 0.48 \times 10^{-4}$ cm^3 mol g^{-2}.

Absorption electron spectroscopy

The absorption spectra of aqueous solutions of the copolymer loaded with tocopherol were recorded using the 'SPEKS SPP-705-1' spectrometer. Quartz cuvettes of 0.2, 0.5, and 1 cm thick were used for measurements.

To determine the K_{ef} value, the dependence $1/(A-A_0)$ was plotted, where A and A_0 are the optical densities at the maximum of the absorption band at a wavelength of 290 nm of encapsulated TP and copolymer, respectively, on the value of 1/[TP]. The ratio of the segment on the ordinate to the slope of this dependence corresponded to the effective binding constant. It is assumed that the interaction between the ligand L and the substrate S is 1 : 1; for this reason a single complex SL (1 : 1) is formed. It was also assumed that the sites (and all the binding sites) are independent and all species obeyed the Beer's law. A wavelength is selected at which the molar absorptivities ε_S (molar absorptivity of the substrate) and ε_{11} (molar absorptivity of the complex) are different. The details and corresponding expression used are given in ref. 23.

IR spectroscopy

FTIR spectra tocopherol and copolymer powders and polymer composition were recorded on the FTIR Bruker ALPHA spectrophotometer in the range of 4000−400 cm^{-1}, the number of scans, 16.

Quantum-chemical calculations of the structure of the tocopherol complex with the copolymer site

The optimized geometry of the tocopherol molecule and the structure of its H-complex with the copolymer site were obtained by quantum chemical modeling performed using Gaussian09, tpssh/6-31G*.[S2]

Calculation of the van der Waals volume of TP molecule

A = 8.405Å   B = 26.045Å   C = 8.836Å
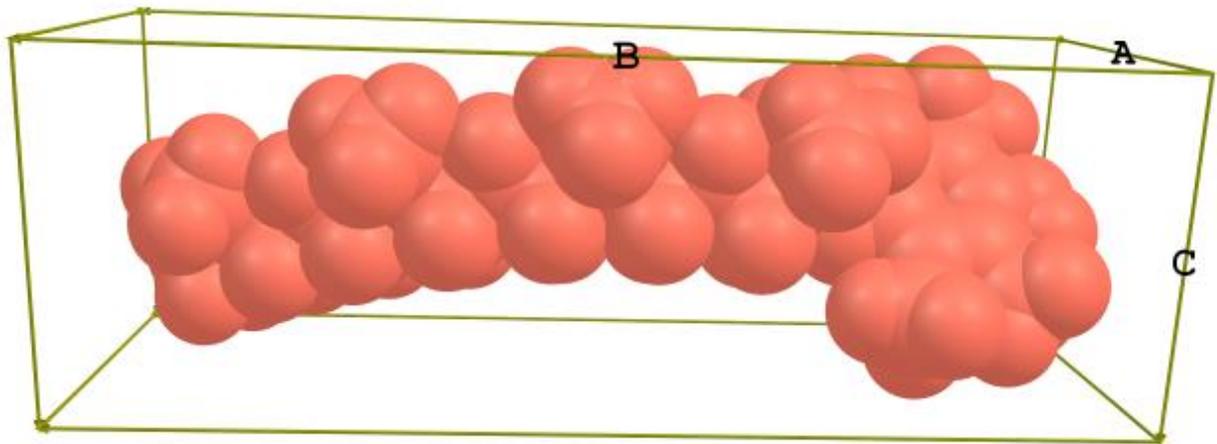
V = 1934.273 Å

Determination of the intensity of lipid peroxidation in the brain homogenate of mice in vitro by the content of malondialdehyde in samples

MDA is one of the most prominent secondary products of lipid peroxidation. The reaction of MDA with thiobarbituric acid (TBA) has been widely used as a sensitive method for LP assay in animal tissues.[S3]

The procedure was performed in accordance with the minor modifications Ohkawa et al. method.[S4] Briefly, mouse brain homogenate in PBS (0.1 M, pH 7.2) was incubated for 30 min at 37 °C with tested conjugates, and the reaction was terminated by addition of 0.4 mL of 17% (w/v) trichloroacetic acid. Following centrifugation for 20 min at 1300×g, 0.5 mL of 0.8% (w/v) of TBA (Sigma-Aldrich) was added to 1 mL of supernatant, heated for 30 min at 95°C and then cooled to RT. The optical density of the TBARS, which corresponds to the produced MDA, was measured at 532 nm against a blank using an Agilent Cary 60 UV-Vis spectrophotometer (Agilent, Santa Clara, United States). The MDA concentration was calculated using an extinction coefficient of 1.56×10^5 $M^{-1}cm^{-1}$.

Statistical processing of the research results was carried out using the statistical software package Microsoft Office Excel, determining the reliability of differences by Student's t-test. Differences between mean values were considered significant at a 99% significance level ($p < 0.001$). Results are presented as mean ± standard error of arithmetic mean (SEM)

Chemical structure of VP-TEGDM copolymer
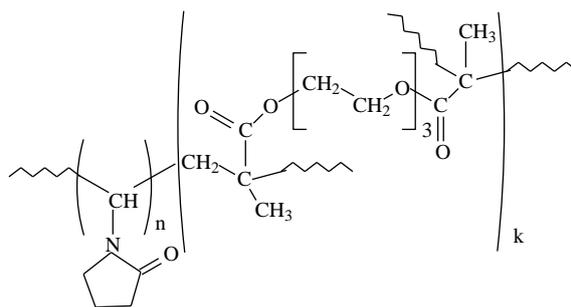
Scheme S1 Chemical structure of VP-TEGDM copolymer.

Table S1 Concentrations of reagents and conditions for encapsulating tocopherol in polymer particles

[Copolymer] in Pr^{i}OH/mg ml^{-1}	[TP] in Pr^{i}OH/mg ml^{-1}	Volume ratios of solutions copolymer and TP/mL	TP content per copolymer/ %	[Copolymer] in water buffer/mg ml^{-1}	[TP]×10^4 in water buffer/M
0.35	0.057	4 : 0	0	0.175	0
		4 : 0.3	1.2		0.1
		4 : 0.6	2.4		0.2
		4 : 1.2	4.8		0.4
		4 : 1.8	7.3		0.6
		4 : 2.4	9.6		0.8

Table S2 Reagent concentrations and conditions for encapsulating tocopherol in polymer particles

[Copolymer] in Pr^{i}OH/mg ml^{-1}	[TP] in Pr^{i}OH /mg ml^{-1}	Volume ratios of solutions copolymer and TP/ml	TP content per copolymer/%	[Copolymer] in water buffer/mg ml^{-1}	[TP]$\times 10^4$ in water buffer/M
3.0	1.14	4 : 0	0	1.5	0
		4 : 0.15	1.25		0.5
		4 : 0.3	2.5		1.0
		4 : 0.6	5.0		2.0
		4 : 0.9	7.5		3.0
		4 : 1.2	10.0		4.0

Table S3 Reagent concentrations and conditions for encapsulating tocopherol in polymer particles

[Copolymer] in Pr^{i}OH /mg ml^{-1}	[TP] in Pr^{i}OH /mg ml^{-1}	Volume ratios of solutions copolymer and TP/ml	TP content per copolymer /%	[Copolymer] in water buffer/mg ml^{-1}	[TP]×10^4 in water buffer/M
7	1.14	4 : 0	0	3.5	0
		4 : 0.15	0.6		0.5
		4 : 0.3	1.2		1.0
		4 : 0.6	2.3		2.0
		4 : 0.9	3.5		3.0
		4 : 1.2	4.9		4.0

Table S4 Influence of TF_{sol}, copolymer, and TP on the content of the product of spontaneous lipid peroxidation – malondialdehyde (MDA) in the mouse brain homogenate *in vitro*

Compound	[Compound], M	MDA content, %
Control	-	100 ± 2
TP_{sol}	10^{-3}	$49\pm3^{**}$
TP_{sol}	5×10^{-4}	$63\pm3^{**}$
copolymer	10^{-3}	101 ± 2
copolymer	5×10^{-4}	98 ± 3
TP^{*}	10^{-3}	$32\pm3^{**}$
TP^{*}	5×10^{-4}	$42\pm3^{**}$
Ethanol	10%	98 ± 2

Note. Solvent - water, *ethanol. Control – mouse brain homogenate without the addition of the test compounds (in the same volumes, a buffer solution was added). Results are presented as a percentage relative to control ± SEM. All measurements were performed in five replicates for three independent experiments. ** − the data are statistically significant (Student's t-test) in relation to the control, $p < 0.001$.

DLS curve of the copolymer

Legend:
- 25°C
- 30°C
- 35°C
- 40°C
- 45°C
- 50°C
- 55°C

X-axis: R_h, nm
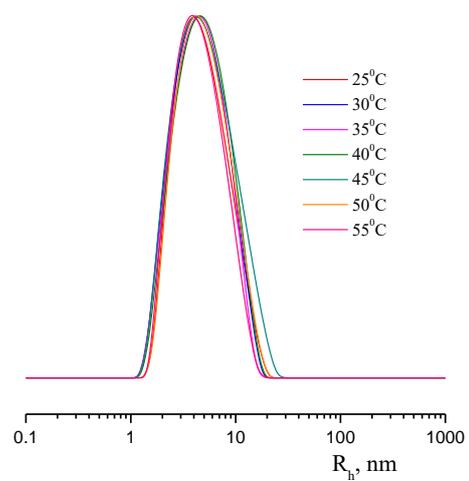
Figure S1 Curves of light scattering intensity distribution over the size of scattering centers with the copolymer solution in Pr^iOH (7 mg mL^{-1}) at different temperatures.

IR spectra of tocopherol, copolymer and copolymer composition
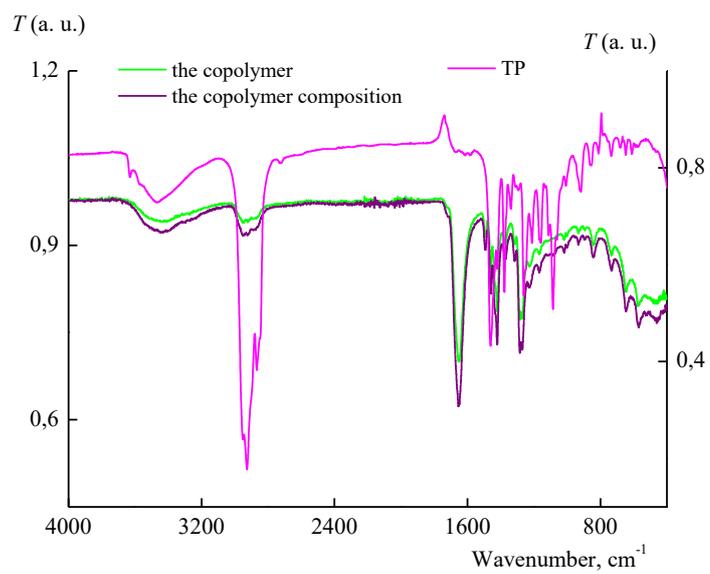
Figure S2 IR spectra of tocopherol, copolymer and copolymer composition.

Absorption spectra of tocopherol solutions

(a)

(b)
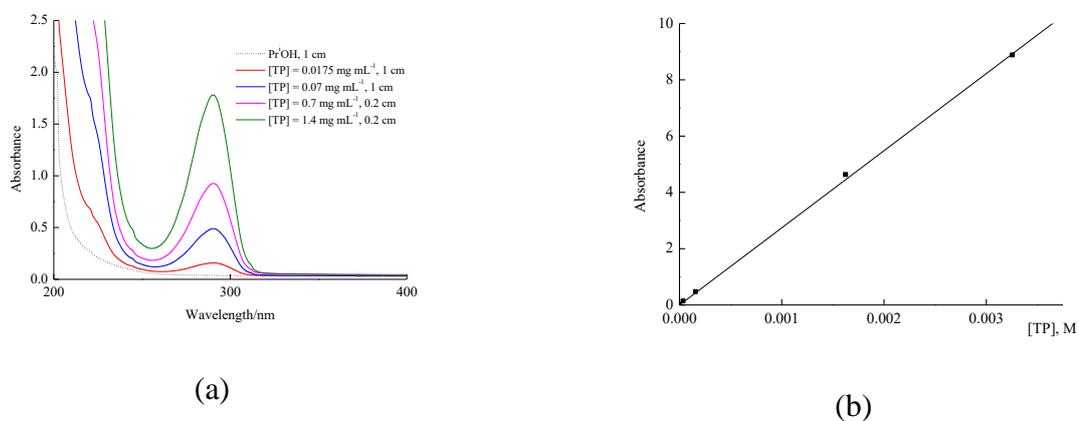
Figure S3 Absorption spectra of solutions of TP in Pr^{i}OH (*a*) and dependence of the optical density of the absorption band of TP at the wavelength with the maximum of 290 nm on its concentration (*b*).

Absorption spectra of the aqueous buffer solution of TP in control experiments
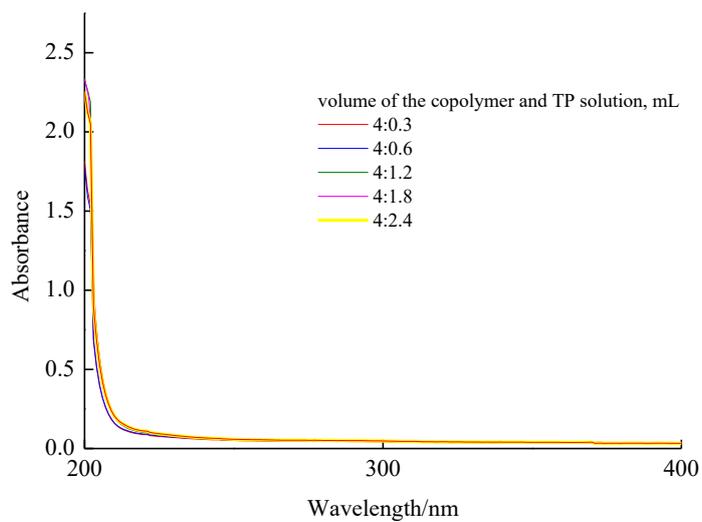
Figure S4 Absorption spectra of the aqueous buffer solution of tocopherol in control experiments (in the absence of copolymer). Cuvette – 1 cm.

Absorption spectra of encapsulated tocopherol in polymer particles
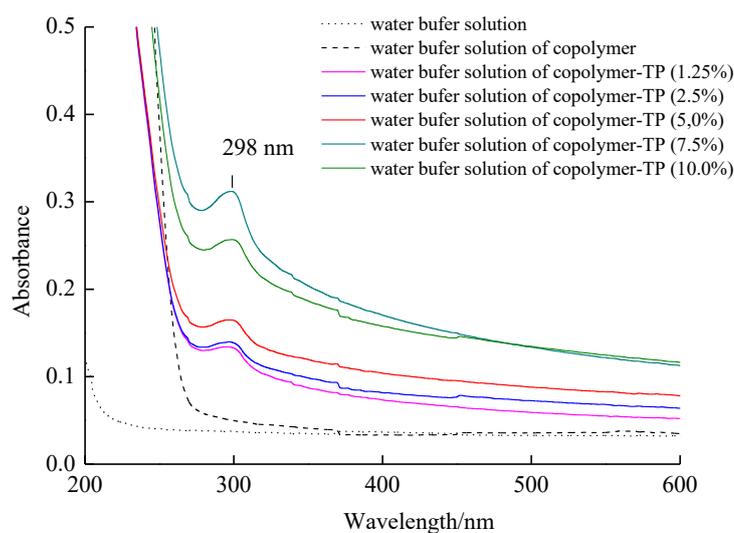
(a)
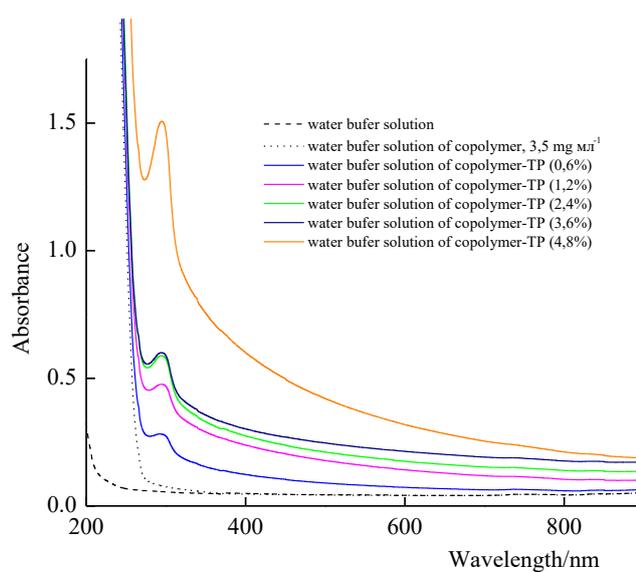
(b)

Figure S5 Absorption spectra of $T\Phi_{sol}$ in an aqueous buffer solution with pH 7.4: (*a*) [copolymer]$_{buffer}$ = 1.5 mg mL^{-1}, cuvette – 0.2 cm. Encapsulation conditions: [copolymer]$_{Pr^iOH}$ = 3 mg mL^{-1} and [TP]$_{PriOH}$ = 1.14 mg mL^{-1}; (*b*) [copolymer]$_{buffer}$ = 3.5 mg mL^{-1}, cuvette – 0.2 cm. Encapsulation conditions: [copolymer]$_{Pr^iOH}$ = 7 mg mL^{-1} and [TP]$_{Pr^iOH}$ = 1.14 mg mL^{-1}.
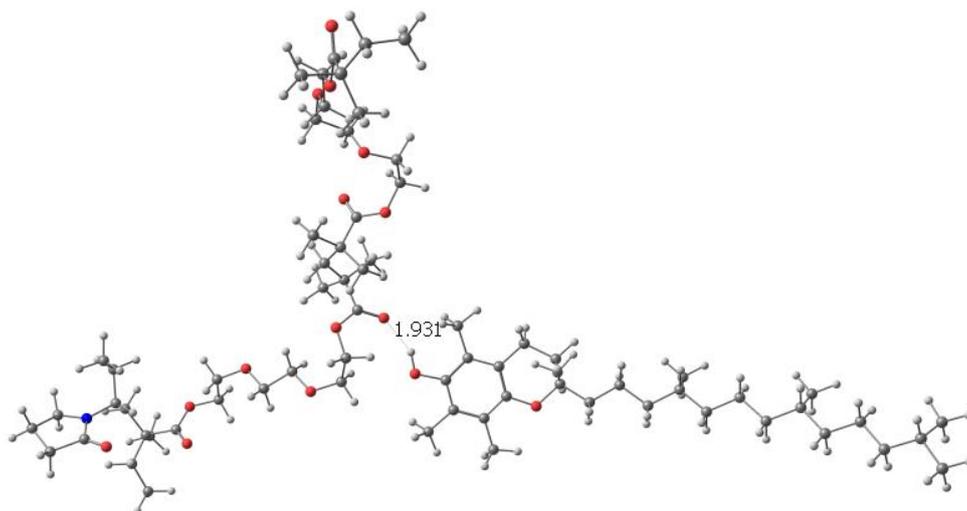
Figure S6 Optimized geometry of the structure of TP with the VP-TEGDM-TEGDM polymer moiety.
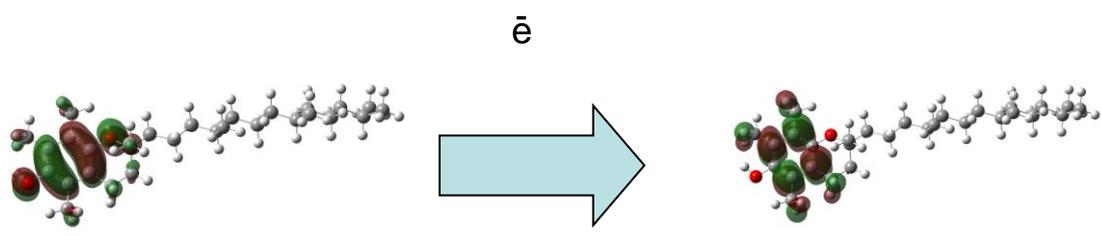
Figure S7 Electronic transition $\pi \to \pi^*$ for TP ($\lambda = 275$ nm) in the aromatic ring [HOMO (TP) → LUMO (TP)].
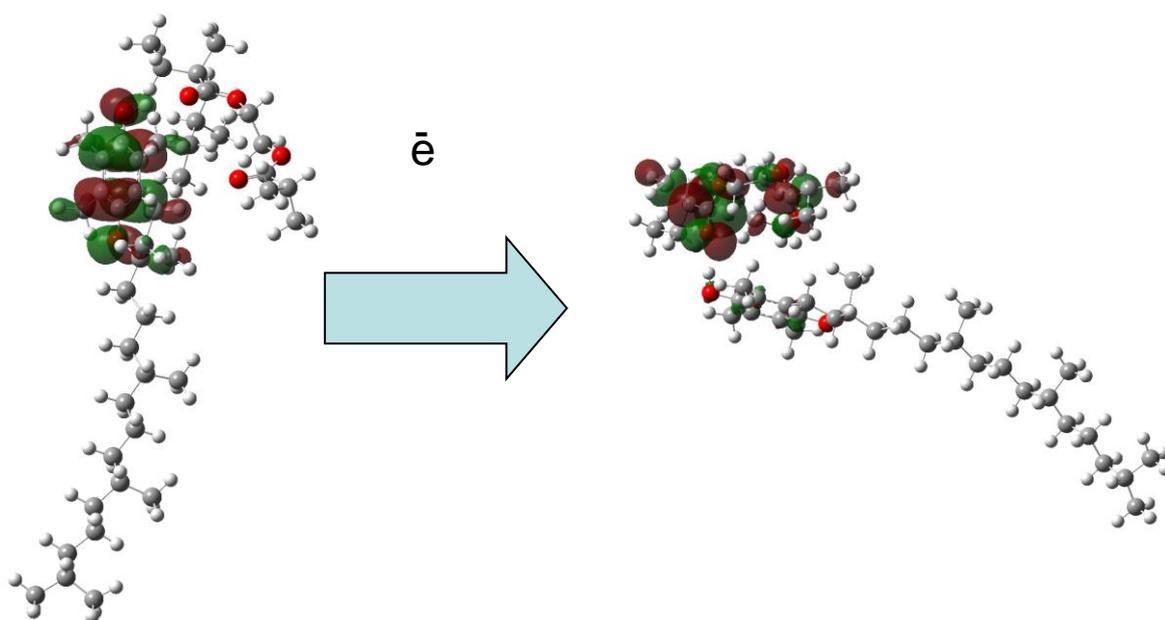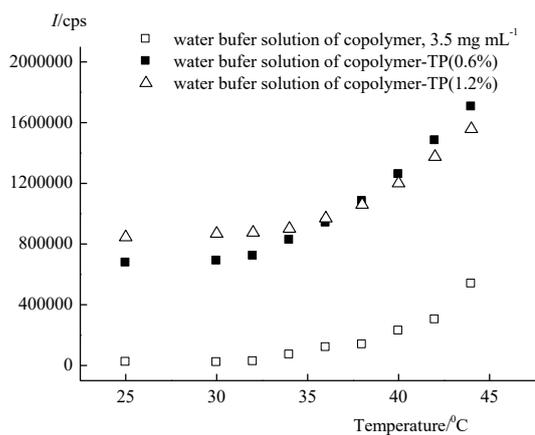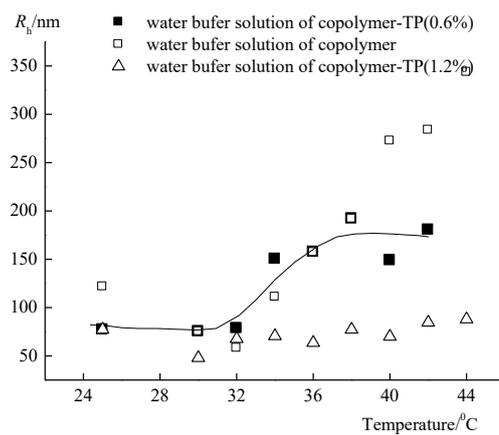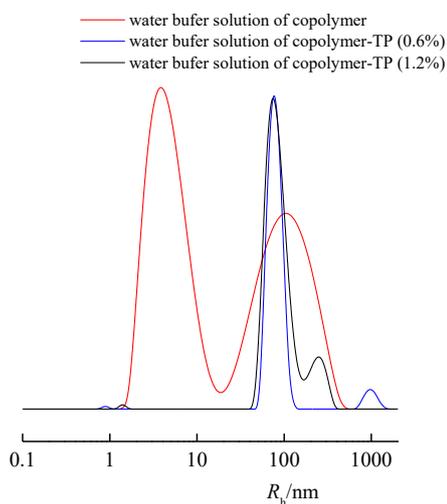
Figure S8 Electronic transition for the TP with VP-TEGDM-TEGDM moiety complex ($\lambda = 270$ nm) from the HOMO TP to the π^* orbital of the polymer moiety.
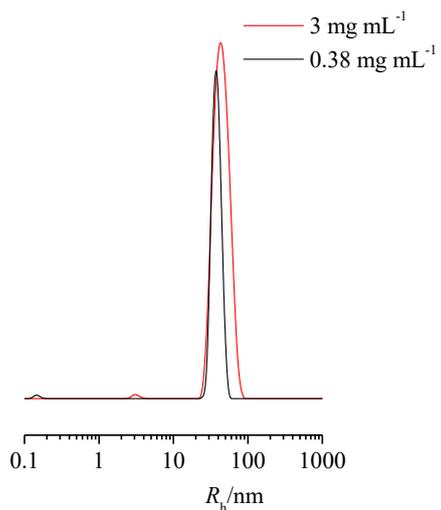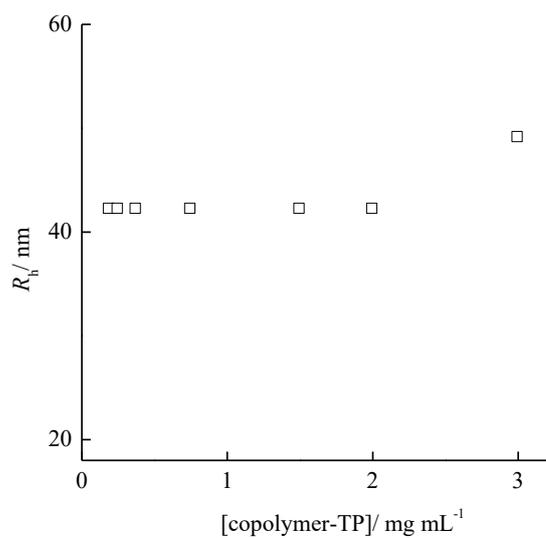
Figure S9 Dependence of the intensity of light scattering by aqueous buffer solutions (a) and hydrodynamic radius (b) on temperature; light scattering intensity distribution over the sizes of scattering centers of the initial copolymer and TP nanostructures at 25 °C (c). [Copolymer]$_{buffer}$ = 3.5 mg mL^{-1}. Encapsulation conditions: [Copolymer]$_{Pr^iOH}$ = 7 mg mL^{-1} and [TP] $_{Pr^iOH}$ = 1.14 mg mL^{-1}.

(a)

(b)

Figure S10 The light scattering intensity distribution over the sizes of scattering centers of the TP nanostructures water solutions at 25 °C (a); the dependence of hydrodynamic radius on TP nanostructures concentration in water solution (b).

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