Microgels based on carboxymethylcellulose as multifunctional carriers for immobilization of inhibitor and activator of inducible NO synthase

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Three-component (ternary) microgels based on carboxymethylcellulose and copper(II) ions with the immobilized inhibitor of inducible NO synthase (*N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl)benzamide), as well as the activator of this enzyme (*N*-(4-isopropylphenyl)-*N*-(1-iminoethyl)piperidine-1-carbothioamide hydrobromide) were prepared. The hydrodynamic and electrokinetic characteristics of the microgels were studied and demonstrate an optimal size and high colloidal stability in both aqueous and physiological environments. The synthesized ternary microgels are characterized by high cytotoxicity causing apoptosis of leukemic *Jurkat* cells.

Key words: carboxymethylcellulose, microgels, copper ions, NO synthase effectors, early apoptosis, *Jurkat* cell line.

The mechanism of formation of binary microgels carboxymethylcellulose—copper ions (CMC—Cu) due to the formation of electrostatic contacts of copper(II) ions with carboxyl groups of the polymer was studied earlier.[1] The negative charge of the prepared water-soluble microgels was determined. The sizes and cytotoxicity of these nanocarriers were shown to depend strongly on the copper content, and rather significant therapeutic window between the effect of microgels on healthy and leukemic cells is observed in some cases. The obtained results provide a possibility of using such carriers for complex delivery of several drug components, including radionuclides and antitumor drugs, *i.e.*, for the preparation of ternary microgels.

Among antitumor agents of various structures,[2,3] inhibitors and activators of NO synthase (NOS) should be distinguished,[4] and among the latter *N*-(5,6-dihydro-4*H*-1,3-thiazin-2-yl)benzamide (inhibitor of inducible NOS (iNOS), L^1) *N*-(4-isopropylphenyl)-*N*-(1-iminoethyl)piperidine-1-carbothioamide hydrobromide (activator of NOS, L^2) have good parameters. 2-Aminopyridine (AP) was also used as a model ligand, and many antileukemic drugs based on AP were developed.

The purpose of this work is to prepare water-soluble ternary microgels CMC—Cu—L^1 and CMC—Cu—L^2 and to study their colloidal stability in aqueous and physiological media and the efficient size and cytotoxicity.

Results and Discussion

Ligand L^1 was introduced into the previously described[1] binary systems CMC—Cu (**I—III**), where the molar ratio Q_1 = [Monomeric unit of CMC] : : [Cu^{2+}] was varied from 7 : 1 to 15 : 1, in such a way that the CMC : L^1 ratio would be 2 : 1 (Table 1). Microgels **IV—VI** were formed, which are highly

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Table 1. Quantitative ratio of the reagents in the ternary nano/microgels CMC—Cu^{II}—L^1

Sample	[Monomeric unit of CMC] : Cu^{2+} (mol.)	*N*/mol		
		CMC	Cu^{II}	L^1 (initial)
IV	15 : 1	$2 \cdot 10^{-4}$	$1.33 \cdot 10^{-5}$	10^{-4}
V	10 : 1	$2 \cdot 10^{-4}$	$2.00 \cdot 10^{-5}$	10^{-4}
VI	7 : 1	$2 \cdot 10^{-4}$	$2.86 \cdot 10^{-5}$	10^{-4}

Table 2. Quantitative ratio of the reagents in the binary and ternary nano/microgels containing L^2

Sample	[Monomeric unit of CMC] : Cu^{2+} (mol.)	*N*/mol		[CMC] : [Cu]	[CMC] : [L^2]
		Cu^{2+}	L^2		
CMC–L^2 (**VII**)	$2 \cdot 10^{-4}$	0	$5 \cdot 10^{-5}$	—	4 : 1
CMC–Cu^{2+}–L^2 (**VIII**)	$2 \cdot 10^{-4}$	$2.00 \cdot 10^{-5}$	$5 \cdot 10^{-5}$	10 : 1	4 : 1

soluble in water, unlike the ligand itself that is nearly insoluble in aqueous solutions.

The content of the ligand in microgels **IV—VI** was determined by spectrophotometry. The UV spectra of ligand L^1 in the prepared samples **IV—VI** are presented in Fig. 1.

The samples with ligand L^2 were synthesized similarly: binary CMC—L^2 and ternary CMC—Cu—L^2 (**VII** and **VIII**, respectively) (Table 2). The corresponding UV spectra are shown in Fig. 2.

The hydrodynamic and electrokinetic characteristics of the synthesized microgels are given in Tables 3 and 4. The EPM (electrophoretic mobility) values indicate a partial neutralization of polysaccharide due to the formation of bridging electrostatic contacts between the macromolecule fragments *via* copper ions, which accompanies the formation of the microgel structure.

An analysis of the physicochemical characteristics (see Table 3) of the microgels containing ligand L^1 shows that the conjugate sizes decrease with an increase in the amount of copper ions, and this contraction processes is more pronounced in a physiological solution. Earlier, when AP was introduced into the binary microgel, the contraction ranged from 400 nm for pure CMC to 350 nm for the ternary microgel. The hydrodynamic diameter of the binary CMC—Cu (7 : 1) microgel was 380 nm in an aqueous solution, and a tendency was observed for decreasing it to 220 nm in a physiological solution and to 195 nm if BSA was additionally introduced.[1] A comparison of two- and three-component compositions **VII** and **VIII** containing ligand L^2 as a NOS activator (see Table 4) shows that the stability of the microgels increases, probably, due to an increase in the number of binding sites of CMC with ligand L^2.

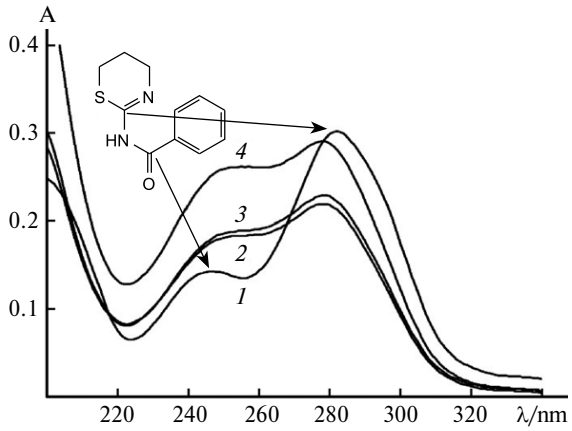

Fig. 1. UV spectra of ligand L^1 (*1*) and complexes **IV—VI** (*2—4*, respectively).

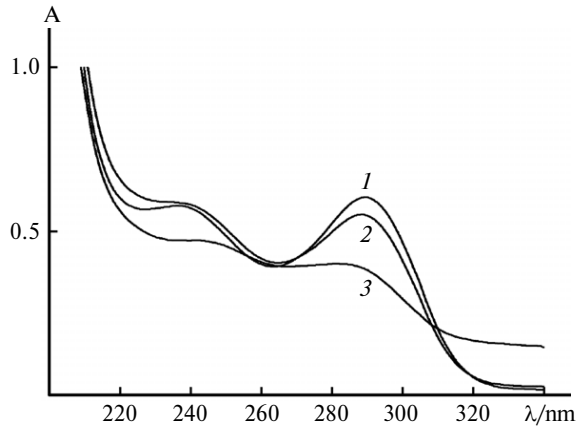

Fig. 2. UV spectra of ligand L^2 (*1*) and complexes **VII** and **VIII** (*2* and *3*, respectively).

Table 3. Hydrodynamic and electrokinetic characteristics of microgels **IV—VI** and aqueous and physiological solutions

Sample	[CMC] : [L^1] (mol.)	D_h*/nm		EPM**
		Aqueous solution	0.15 *M* NaCl	
IV	2 : 1	370±25	285	−(3.34±0.05)
V	2 : 1	340±25	230	−(3.31±0.02)
VI	2 : 1	230±15	190	−(3.46±0.02)

* Hydrodynamic diameter.
** In μm cm s^{-1} V^{-1}.

Table 4. Hydrodynamic and electrokinetic characteristics of aqueous solutions of microgels **VII** and **VIII***

Sample	D_h/nm		EPM**
	Aqueous solution	0.15 *M* NaCl	
VII	220	110	−5.99
VIII	250	180	−4.86

* In all cases, the concentration of aqueous solutions of the microgels was 1 mg mL^{-1}.
** In μm cm s^{-1} V^{-1}.

Owing to this, the hydrodynamic sizes decrease over those of the microgels containing both AP and L^1. In a physiological solution the size of these particles approach that of 0-dimensional nanoparticles, which is a very important parameter for medical remedies.[5,6]

Flow cytofluorimetry to late apoptosis and necrosis was used to evaluate the cytotoxic effect of the microgels on the cells (Fig. 3). As shown previously,[7] a tendency for increasing early apoptosis was found for both two- and three-component microgel containing AP, and the higher the content of copper ions in the samples, the stronger the tendency. A resembling pattern of cell death of the *Jurkat* cell line is observed for the samples containing ligands L^1 and L^2 in the microgels. The radiation stability of the ligands was considered with allowance for the further

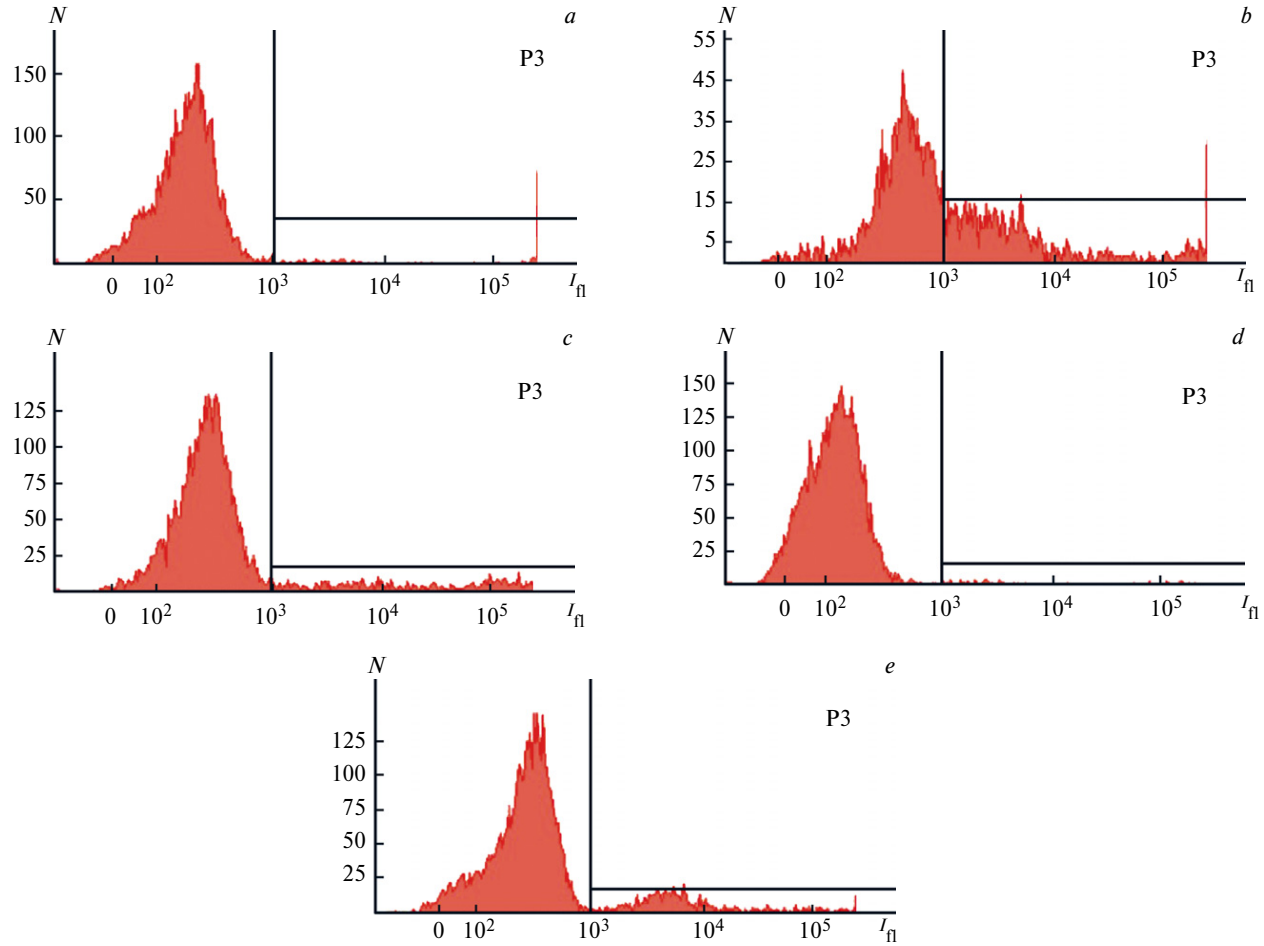

Fig. 3. Analysis of cell death of the *Jurkat* cell line: negative control (*a*), positive control in the presence of staurosporine (*b*), in the presence of complexes **VIII** (*c*), **VII** (*d*), and **IV** (*e*); *N* is the number of cells, I_{fl} is the fluorescence intensity, and P3 are the cells at the late apoptosis stage.

introduction of copper radionuclides into the microgels. It turned out that under γ-irradiation the stability of the ligands in a physiological solution is retained to a dose of approximately 1.5 kGy.

Experimental

Ligands L^1 and L^2 were synthesized using previously described procedures.[4,8,9]

Microgels IV—VIII. Ternary microgels were synthesized by the introduction of the corresponding ligand into CMC—Cu (CMC: M = 90 kDa, degree of substitution φ = 0.7) in such a way that the molar ratio CMC : ligand would be 2 : 1 as described earlier.[1] Binary microgels Cu^{II}—CMC were synthesized at ambient temperature: a solution (5 mL) of $CuSO_4 \cdot 5\ H_2O$ (from 3.7 to 7.4 mg) was added dropwise with vigorous stirring to a 0.1% solution (50 mL) of CMC. The ratio Q = [Monomeric unit of CMC] : $[Cu^{2+}]$ = 15 : 1 (**IV**), 10 : 1 (**V**), and 7 : 1 (**VI**) was thus varied increasing the Cu^{2+} content in the reaction mixture. The prepared solutions were stirred for 20 min, after which dialysis was carried out for 1 day (Sigma, MWCO (molecular weight cut-off) ~12000 Da) to wash out low-molecular-weight salts. After dialysis, the solutions were lyophilically dried. The yield of the dry product was ~90 wt.%.

Hydrodynamic radius/diameter was determined by the dynamic light scattering method on an ALV-5 instrument (ALV, Germany) equipped with a He—Ne laser with a power of 25 mW and a wavelength of 632.8 nm (scattering angle 90°). Mathematical processing was performed by Tikhonov′s regularization method. Hydrodynamic radii were calculated by the Stokes equation: $R_h = kT/(6\pi\eta D)$, where R_h is the Stokes radius of the particle, k is Boltzmann′s constant, T is the temperature of the mixture, η is the viscosity of the medium, and D is the diffusion coefficient in a given medium. The measurements were repeated up to 10 times.

Electrokinetic characteristics (EPM) were determined on a Brookhaven Instruments analyzer of electrophoretic scattering and nanoparticle sizes (NanoBrook Omni, USA).

Quantitative measurements of copper ions and ligands in the samples were carried out spectrophotometrically (Shimadzu UV-1280, Japan) using preliminarily obtained calibration curves.

Flow cytofluorimetry. The method is based on the laser irradiation of a cell flow and further detection of fluorescence and scattered light of each cell. To color cells, propidium iodide (Sigma—Aldrich, USA), which can penetrate into the cell only through the damaged membrane of the cells at the later apoptosis stage or death cells, was used in a concentration of 1 μg mL^{-1} was used. A FACSAria cytofluorimeter (BD Bioscience, USA) with fluorescence excitation with an argon laser (λ = 488 nm) was used for analysis of the samples. The data were processed using the Statistica 5.11.2 program.

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Animal Testing and Ethics

No human or animal subjects were used in this research.

Conflict of Interest

The authors declare no competing interests.

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