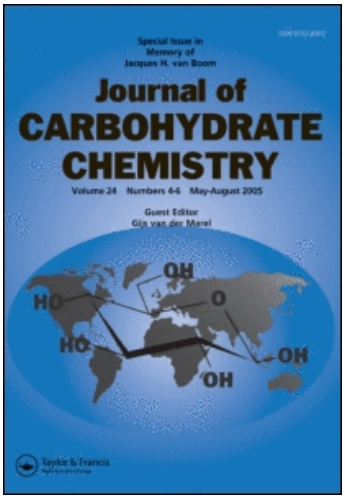

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Copolymers of 2-Deoxy-2-Methacrylamido-D-Glucose with Aminoacrylates and Allylamine Hydrochloride

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New polyvinylsaccharides containing primary or tertiary amino groups were synthesized by radical copolymerization of 2-deoxy-2-methacrylamido-D-glucose (MAG) with 2-(dimethylamino)ethyl methacrylate, 2-(diethylamino)ethyl methacrylate (DEAEM), or allylamine hydrochloride. The reactivity ratios of comonomers were determined. The effect of copolymer composition on the conformational properties of macromolecules was detected. For MAG-DEAEM copolymers with DEAEM unit content more than 60 mol-% the increase of degree of ionization causes conformational transition at $\alpha = 0.3$ to 0.7 from compact polymer coil to loose coil due to the electrostatic repulsion of charged amino groups. The increase of MAG content in copolymers is accompanied by a decrease of their cytotoxicity.

Keywords Copolymerization; Polyamines; Polyvinylsaccharides; Water-soluble polymers

INTRODUCTION

The specific molecular and cell recognition ability of glycopolymers makes it possible to use them for biomedical applications. The intense work is being carried out today concerning the synthesis and study of new glycopolymers.[1–6]

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A great number of different sugar-containing synthetic polymers with different structures have been described in literature. The linear, comb polymers, dendrimers, cross-linked, and other types of carbohydrate-containing polymers are known now. Among them, water-soluble polyvinylsaccharides attract considerable interest. Homo- and copolymers of unsaturated derivatives of glucose, galactose, mannose, sorbose, lactose, and other saccharides varying in the type of saccharide-polymer chain linkage and position of glucose ring substitution have been obtained by polymerization of appropriate monomers.[1–16] N-vinylpyrrolidone, acrylamide, N-isopropylacrylamide, N-vinylformamide, and other comonomers have been used. Various sugar residues were introduced to poly-N-(2-hydroxypropyl)methacrylamide, polyallylamine hydrochloride, polylysine, polyethylene imine, and other polymers as a result of polymeranalogous reactions.[1,4,17–19]

Polyvinylsaccharides containing ionogenic groups are promising for some biological applications.[17,20] They were obtained by polymer analogous reactions of neutral polyvinylsaccharides or by polymerization of vinylsaccharide and ionic monomer.[1,20] The capacity of glycopolymers to bind proteins was used for protein stabilization, and it has been found that introduction of cationic or anionic groups to polyvinylsaccharides by polymeranalogous reactions resulted in a greater ability of ionic polymers to stabilize horseradish peroxidase than neutral precursor.[20] The efficacy of stabilization depends on the sugar nature and the type and content of ionic groups. Cationic polyvinylsaccharides were successfully used for delivery of DNA to cells.[17] In this case the nature of carbohydrate, its content, and the cell type have an effect on efficiency of DNA transfer.

Hydrophobicity of macromolecule is an important characteristic that can control interaction of polymer with cell membranes.[21,22] The use of comonomers possessing different hydrophobicity (e.g., N-vinylacetamide, N-butyl-N-vinylacetamide, N-octyl-N-vinylacetamide) makes it possible to control the hydrophobicity of sugar-containing polymer.[9]

The present work deals with the synthesis and investigation of new copolymers of 2-deoxy-2-methacrylamido-D-glucose (MAG) and monomers bearing primary or tertiary amino groups and differing in their pK_a values and hydrophobicity—allylamine hydrochloride (AA), 2-dimethylaminoethyl methacrylate (DMAEM), and 2-diethylaminoethyl methacrylate (DEAEM). Cytotoxicity of copolymers was studied for preliminary decision about the ability of their use for biomedical applications.

EXPERIMENTAL PART

(+)–D-glucosamine hydrochloride, allylamine, DMAEM, DEAEM, 2,2′-azobisisobutironitrile (AIBN), 2,2′-azobis(2-methylpropionamidine) dihydrochloride

(AMPA), and dimethylformamide (DMFA) were purchased from Aldrich (Germany). Allylamine was distilled and DMAEM, DEAEM, and DMFA were distilled under reduced pressure before use.

Allylamine hydrochloride (60 wt.-% water solution) was received according to reference 23. MAG was synthesized according to reference 24.

The free radical copolymerizations of MAG with comonomers were carried out in sealed ampoules under argon at 60°C in DMFA for 24 h (MAG-DMAEM, MAG-DEAEM) or at 50°C in water solution (MAG-AA). Concentration of monomers was 10 wt-%. AIBN (2 wt-% of monomers content) was used as a radical initiator for MAG-DMAEM and MAG-DEAEM systems and AMPA (5 wt-% of monomers content) as an initiator for MAG-AA. Copolymers were dialyzed through a 1,000-molecular-weight cutoff dialysis tubing (Spectra/Por 7, USA) against 2% NaCl water solution for 24 h and against water for 24 h. Then copolymers were lyophilized. The absence of low molecular impurities in polymer samples was confirmed by thin layer chromatography in all cases.

In reactivity ratio experiments the yield of polymers did not exceed 5%.

The compositions of the copolymers of MAG and DMAEM or DEAEM were determined by potentiometric titration of DMAEM or DEAEM units. The compositions of the copolymers of MAG and AA were determined (1) from the absorption of complexes of AA units and 2,4,6,-trinitrobenzenesulfonic acid, $\lambda_{max} = 420$ nm[25] and (2) by potentiometric titration of allyl hydrochloride by $AgNO_3$ solution. Results of both methods coincided.

Intrinsic viscosity values were determined in 0.1 M Na_2SO_4 solution for water-soluble copolymers or in DMFA solution for polymers that could not be dissolved in water. Molecular masses (MM) of copolymers containing more than 80 mol-% MAG were estimated viscometrically using Mark-Kuhn-Houwink parameters found for poly-MAG:

$$[\eta] = 8.29 \times 10^{-4}\, M^{0.49}\ (25^{\circ}C\ 0.1\ M\ Na_2SO_4).^{[7]}$$

The curves of potentiometric titration for a weak polymer base are described by the Henderson-Hasselbach equation[26]:

$$pH = pK_0 - \log \alpha/(1-\alpha) + 0.43\Delta G_{el}/RT,$$

where pK_0 is a dissociation constant of ionizable group at the absence of electrostatic interactions, ΔG_{el} is a free electrostatic energy change resulting from electrostatic interaction of ionized groups of macromolecule, and α is a degree of ionization of amino groups. The value of α was determined as a ratio of ionized amino group concentration and the total concentration of amino groups in solution. The added quantity of HCl was assumed to be equal to ionized amino group concentration.

UV spectra were obtained on a Specord M-40 spectrophotometer. ^{1}H NMR spectra were recorded on a Bruker Avance 400 spectrometer in D_2O.

For determination of polycation cytotoxicity, viability of T-98G cells (cells of human astrocytome) in the presence of polymer was evaluated in a microtetrazol test (MTT).[27] IC_{50} concentrations (concentrations of polymers inhibiting 50% of cells) were determined.

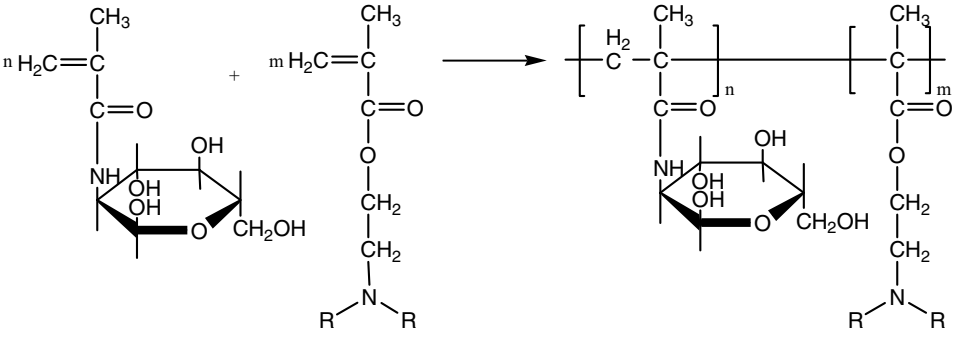
RESULTS AND DISCUSSION

MAG-DMAEM and MAG-DEAEM Copolymers

Radical copolymerizations of MAG and aninoacrylates DMAEM or DEAEM were carried out according to Scheme 1:

The characteristics of MAG-DMAEM and MAG-DEAEM copolymers obtained for different compositions of initial monomer mixtures are given in Table 1. For all initial monomer ratios the rates of copolymerizations of MAG with DMAEM or DEAEM are high. The yields of copolymers after 4 h of copolymerization were more than 60% to 70% and those after 24 h were equal to 90% to 95% in all cases. Compositions of copolymers obtained after 24 h correlate with compositions of initial monomer mixtures (Table 1).

Solubility of MAG-DMAEM and MAG-DEAEM copolymers differ from solubility of appropriate homopolymers. Homopolymer of DMAEM is soluble in water and alcohols; homopolymer of DEAEM is soluble in alcohols, but insoluble in water. Homopolymer of MAG is soluble in water and insoluble in alcohols. All copolymers MAG-DMAEM are water soluble. Copolymers MAG-DMAEM and MAG-DEAEM containing more than 25 mol-% of MAG units are insoluble in alcohols. Copolymers containing more than 30 mol-% of DEAEM are insoluble in water. All polymers are soluble in DMFA and DMSO.



$R = CH_3$ or C_2H_5

MAG DMAEM or DEAEM

Scheme 1:

Table 1: Copolymerization of MAG (M_1) and DMAEM or DEAEM (M_2)

		Monomer feed			Copolymer		
№	M_2	$[M_1]:[M_2]$, mol-%	$[M_1+M_2]$, mas-%	[AIBN], mas-% of $[M_1+M_2]$	$[M_2]$, mol-%	$[\eta]$, dL/g Medium	Value/MM
1	DMAEM	90:10	20	0.5	7.6	0.1 M Na_2SO_4	0.24/105,000
2	DMAEM	90:10	10	2	7.0	0.1 M Na_2SO_4	0.14/35,000
3	DMAEM	75:25	10	2	18.1	0.1 M Na_2SO_4	0.11/21,000
4	DMAEM	50:50	10	2	45.2	0.1 M Na_2SO_4	0.09
5	DMAEM	30:70	10	2	65.1	0.1 M Na_2SO_4	0.09
6	DEAEM	70:30	10	2	27.2	0.1 M Na_2SO_4	0.13
						DMFA	0.12
7	DEAEM	60:40	10	2	36.8	DMFA	Not determined
8	DEAEM	50:50	10	2	48.2	DMFA	0.10
9	DEAEM	25:75	10	2	76.3	DMFA	0.12

For copolymers 1–3 containing less than 20 mol-% of DMAEM units (Table 1), estimation of MM values was made using Mark-Kuhn-Houwink parameters found for poly-MAG.[7] As can be seen from Table 1, the decrease of monomer concentration and increase of AIBN content in the initial mixture resulted in a decrease of intrinsic viscosity and MM values (copolymers 1 and 2).

The analysis of titration curves of copolymers (dependence of $pK_{app} = pH - \log \alpha/(1-\alpha)$ vs α) allowed us to consider the effect of copolymer composition on its electrochemical and conformational properties. It is well known that the shape of the titration curve is sensitive to the conformational state of polyelectrolyte molecules.[26,28] The pK_{app} vs. α dependence of investigated MAG-DMAEM copolymers is characterized by monotonous, nearly linear decreasing of pK_{app} values with α in a range 0.2 to 0.8. Analogous dependence was observed for DMAEM copolymers with another neutral monomer—N-vinylpyrrolidone (VP). Such shape of titration curves, $pK_{app}(\alpha)$, is similar to those for polyelectrolyte molecules, conformation of which changes during titration from statistical coil to extended polymer chains due to the electrostatic repulsion between the charged groups. The pK_{app} values estimated at $\alpha = 0.5$ are listed in Table 2. It can be seen that the increase of content of neutral comonomer MAG or VP is accompanied by increasing of basic properties of amino groups. The pK_{app} values of DMAEM units in copolymers approach to a pK_0 value of their low-molecular-weight model—dimethylaminoethylpyvalate.[29] The dependences of pK_{app} on DMAEM unit content for both copolymers MAG-DMAEM and VP-DMAEM are very close, but pK_{app} values for MAG-DMAEM copolymers are lower than those for VP-DMAEM copolymers (see Table 2).

The pK_{app} (α) curves of MAG-DEAEM copolymers (Fig. 1) exhibit the similar shape, if the DEAEM unit content does not exceed 60 mol-%. The dependence $pK_{app}(\alpha)$ for copolymers with more than 60 mol-% of DEAEM units is

Table 2: Effect of copolymer composition on pK_{app} in aqueous solution at $\alpha = 0.5$

Copolymer	DMAEM, mol-%	pK_{app}
MAG-DMAEM	7	7.81
	45	7.22
	75	6.89
	82	6.81
VP-DMAEM	13	7.40
	17	7.16
	26	7.07
	41	6.86
	75	6.70
Poly-DMAEM	100	6.70
MAG-DEAEM	18	7.82
	31	7.78
	34	7.55
	43	7.52
	48	7.48
	68	7.25
	76	6.98
Poly-DEAEM	100	6.74

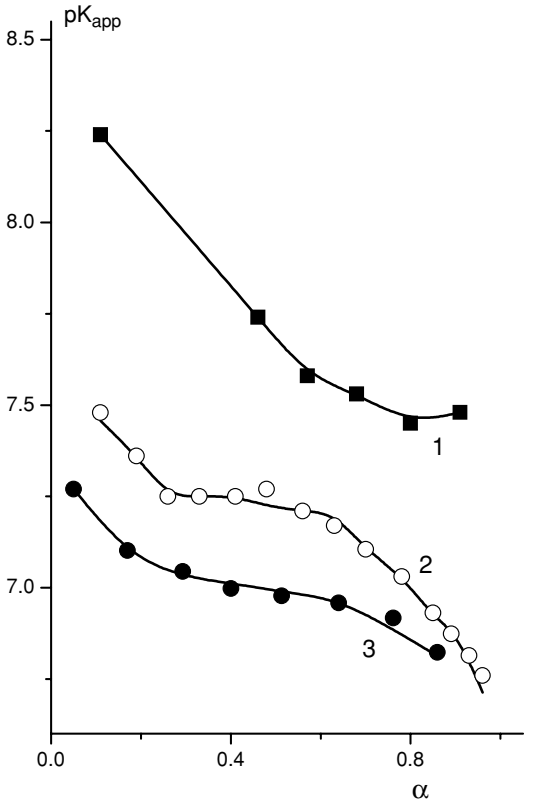


Figure 1: Potentiometric titration curves plotted as $pK(\alpha)$ vs. α for MAG-DEAEM copolymers of various composition. 1–18, 2–68, 3–76 mol-% DEAEM.

characterized by three regions, namely (1) the initial decrease of pK_{app} values, (2) plateau at the α region 0.3 to 0.7, and (3) the slight decrease of pK_{app} values at $\alpha > 0.7$ (Fig. 1). Observed dependence on pK_{app} vs. α can be attributed to the conformational transition in copolymer molecules during ionization of amino groups from compact coil to loose coil.[26,28] At low concentration of charged amino groups the compact structure of polymer coil is stabilized by hydrophobic interactions of nonpolar C_2H_5 groups of DEAEM units. With increasing quantity of protonated amino groups ($0.7 > \alpha > 0.3$), the number of charged groups in polyelectrolyte coil increases and electrostatic repulsion results in unfolding of the compact macromolecular coil. At ionization degree of amino groups $\alpha > 0.7$ (high concentration of protonated amino groups), we have a fully extended polymer chain.

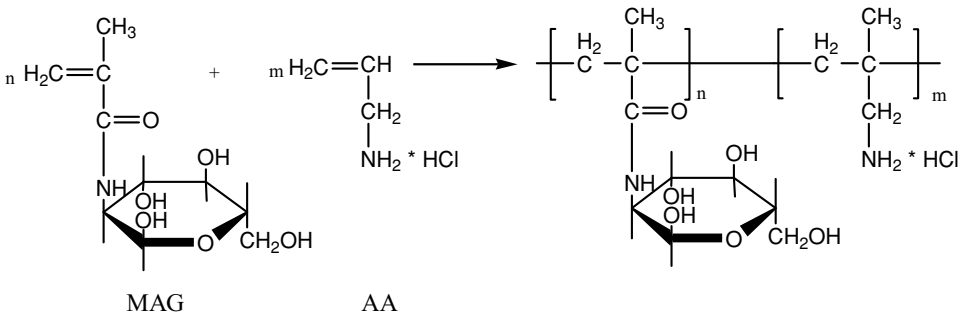
MAG-AA Copolymers

Copolymers MAG-AA were synthesized by radical polymerization according to Scheme 2:

The characteristics of MAG-AA copolymers are given in Table 3. As can be seen, the increase of AA content in the initial monomer mixture from 30 to 70 mol-% results in the decrease of yield, [η] value, and MM of forming polymer. The content of AA units in copolymers obtained is considerably lower than that in the initial monomer mixtures. All that is typical for inactive allylic monomers.[30,31]

Poly(allylamine hydrochloride) is not soluble in DMFA as distinct from poly-MAG. It was found for MAG-AA copolymers that the more copolymer is rich in AA units, the less solubility in DMFA is. Copolymers 10 and 11 are completely soluble, copolymer 12 is partly soluble, and copolymers 13 and 14 are completely insoluble in DMFA. All these polymers are soluble in water.

Figure 2 shows the ^1H NMR spectra of MAG(M)-AA(A) copolymers 12 and 13 and homopolymer of AA. The signal of H11 of copolymers at 2.8 ppm, unlike the one of poly-AA, represents the badly resolved multiplet due to neighbor effect of MAG units on chemical shifts in copolymers.



Scheme 2:

Table 3: Copolymerization of MAG and AA (M_2)

	Copolymerization		Copolymer			
№	$[M_1]:[M_2]$, mol-%	Time, h	Yield, %	$[M_2]$, mol-%	$[\eta]$, dL/g	MM
10	70:30	74	66. 4	6.1	0.19	66,000
11	50:50	72	60.1	14.0	0.13	29,000
12	30:70	74	46.4	22.7	0.11	Notdetermined
13	10:90	19	23	36.4	Not determined	Not determined
14	6:94	25	9.5	52.9	0.05	Notdetermined

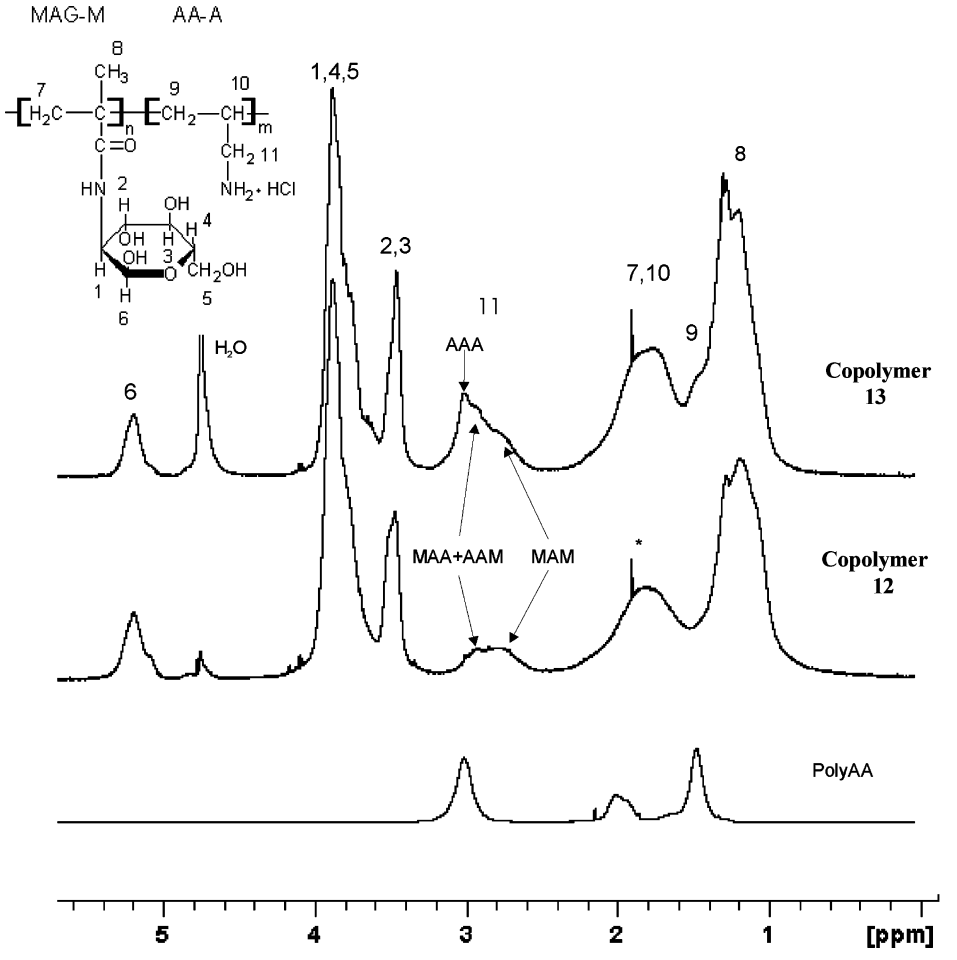


Figure 2: ^{1}H NMR spectra of MAG-AA copolymers of different composition. * impurity peak of residual acetone.

Table 4: Reactivity ratios

M_2	Method: Fineman-Ross	Method: Kelen-Tüdös	Method: Ezrielev-Brokhina-Roskin	Average value
DMAEM	$r_1 = 0.83 \pm 0.21$	$r_1 = 0.85 \pm 0.14$	$r_1 = 0.82 \pm 0.02$	$r_1 = 0.83 \pm 0.21$
	$r_2 = 2.20 \pm 0.13$	$r_2 = 2.25 \pm 0.26$	$r_2 = 2.59 \pm 0.09$	$r_2 = 2.37 \pm 0.26$
DEAEM	$r_1 = 0.83 \pm 0.10$	$r_1 = 0.89 \pm 0.16$	$r_1 = 0.87 \pm 0.02$	$r_1 = 0.87 \pm 0.16$
	$r_2 = 3.74 \pm 0.74$	$r_2 = 3.98 \pm 0.32$	$r_2 = 3.97 \pm 0.40$	$r_2 = 3.93 \pm 0.74$
AA	$r_1 = 17.89 \pm 1.10$	$r_1 = 18.13 \pm 3.06$	$r_1 = 17.42 \pm 1.79$	$r_1 = 17.81 \pm 3.06$
	$r_2 = 0.13 \pm 0.03$	$r_2 = 0.13 \pm 0.05$	$r_2 = 0.14 \pm 0.01$	$r_2 = 0.13 \pm 0.05$

Reactivity ratios

Microstructure of biomedical polymers is an important characteristic. For polymer carriers, for example, it can have an influence on the peculiarities of interaction of polymer with active substance and on properties of conjugates obtained. Copolymerizations of MAG (M_1) and AA, DMAEM, or DEAEM have not been described earlier, so we estimated the reactivity ratios of monomer pairs investigated.

In all experiments the yield of polymers did not exceed 5%. The reactivity ratios (r_1, r_2) have been calculated according to the general copolymer composition equation by applying the linearization methods suggested by Fineman and Ross,[32] Kelen and Tüdös,[33] and Ezrielev, Brokhina, and Roskin.[34] Table 4 shows the r_1 (MAG) and r_2 values obtained. As can be seen, DMAEM and DEAEM are very similar as to their copolymerization activities. It was found that $r_1 < 1$ and $r_2 > 1$ for both comonomers. The enrichment of forming polymers by aminoacrylate (DMAEM and DEAEM) units as compared to the monomer feeds takes place at the initial stages of copolymerization. On the contrary, for the MAG-AA system, not only are $r_1 > 1$ and $r_2 < 1$, but also $r_1 \gg r_2$ and growing polymer chains at the beginning of process contain much more MAG units than monomer mixtures do. So the microstructure of MAG-AA copolymers must differ greatly from the microstructure of MAG-DMAEM and MAG-DEAEM copolymers.

The found values of r_1 and r_2 don't contradict the methacrylate and methacrylamide reactivity ratios known in literature. There are different values for different pairs of monomers, but $r_1 < 1$ (methacrylate) and $r_2 > 1$ (methacrylamide) are described for many pairs including 1-deoxy-1-methacrylamido-O-glucitol–methyl methacrylate (MMA),[35] DMAEM-MMA, and DEAEM-MMA.[36] For methacrylic acid derivatives and allylic monomers, $r_1 > 1$, $r_2 < 1$, $r_1 \gg r_2$, and r_2 values close to zero were found in all cases.[30,37] We determined some greater value of $r_2 = 0.13$ that can be explained by conditions of copolymerization supporting even homopolymerization of AA.[23]

The values of $r_1 \cdot r_2 > 1$ were found for all systems investigated. We determined $r_1 \cdot r_2 > 1$ for MAG-acrylic acid and MAG-methacrylic acid pairs earlier.[38] Such values of $r_1 \cdot r_2$ were found for other pairs[39–42] and were explained by influence of solvent or substituent. The MAG unit contains bulky saccharide residue that can have an influence on the copolymerization process due to steric hindrances. The interactions of monomers or growing radicals with the solvent DMFA also cannot be eliminated.

As was mentioned above, the signal of H11 of MAG(M)-AA(A) copolymers at 2.8 ppm in the ^{1}H NMR spectra represents the badly resolved multiplet of copolymer triads AAA, AAM, and MAM centered at the AA monomer unit. The composition of the copolymers can be calculated from the ratio of integral intensity of H1-H5 atom signals of pyranose ring (MAG) at 3.4 to 3.8 ppm and that of H11 signal (side CH_2 group of AA) at 2.8 ppm. It was determined that according to ^{1}H NMR spectra, copolymers 12 and 13 contain 20 and 40 mol-% of AA units, respectively. These data are in agreement with data obtained by spectrophotometric and potentiometric methods (Table 3). The relative integral intensity of signal homotriad (AAA) at 3.0 ppm was obtained by deconvolution of the multiplet copolymer signal on components. The run number (R) of copolymer was calculated, using the formula $R = (2 - 2\sqrt{F_{AAA}})N_A$, where F_{AAA} is relative integral intensity of AAA triads in multiplet and N_A is mole fraction of monomer A in copolymer.[43–45] The relative integral intensity of AAA triads for copolymer 13 was 0.36 and calculated experimental R value was 32, which is close to calculated $R = 38$ using the loaded molar monomer ratio MAG:AA 10:90 and relative activities r_1 and r_2 of monomers from Table 4. The average block length of AA in copolymer 13 calculated from experimental R value is equal to 2.5. For copolymer 12 intensity of the signal of AAA triads is very low and allylamine units alternate with MAG units.

For copolymers MAG-DMAEM and MAG-DEAEM, we cannot resolve triad signals in ^{1}H and ^{13}C NMR spectra.

Copolymer Cytotoxicity

The toxicity is one of the prime characteristics of biomedical polymers. Copolymers investigated exhibited low cytotoxicity. Homopolymer of MAG is nontoxic. Polyamines exhibit dose-dependent cytotoxicity, and it was established earlier that for PDMAEM (MM 10,000–1,500,000), values of IC_{50} are equal to 1,7 to 4,5 μg/mL.[46] We found that the increase of MAG content in copolymers is accompanied by a decrease of toxicity, which means that IC_{50} values increase. Thus, IC_{50} of MAG-DMAEM copolymers containing 35 and 93 mol-% MAG (polymers 5 and 9) are equal to 30 and 1,000 μg/mL, respectively.

Preliminary investigation showed that copolymers of MAG and DMAEM, DEAEM, or AA can effectively bind DNA and DNA can be delivered into cells with the help of the complex formed.

CONCLUSION

Thus, new copolymers of 2-deoxy-2-methacrylamido-D-glucose with aminoacrylates and allylamine hydrochloride differing in composition microstructure hydrophobicity and toxicity were synthesized by radical copolymerization. The reactivity ratios of monomer pairs investigated were determined. Analysis of pK_{app} dependence on degree of ionization showed that amino groups in copolymers MAG-DMAEM and MAG-DEAEM behave as a weak base. For MAG-DEAEM copolymers with DEAEM unit content more than 60 mol-%, the increase of degree of ionization causes conformational transition at $\alpha = 0.3$ to 0.7 from compact polymer coil to loose coil due to the electrostatic repulsion of charged amino groups. The copolymers synthesized provide perspective for biomedical use, in particular for DNA delivery into cells.

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