Conjugates of hyaluronic and carboxy hyaluronic acids and their *in vitro* biodegradability upon the action of testicular hyaluronidase

I. Yu. Ponedel´kina, E. S. Lukina, E. A. Khaibrakhmanova, E. V. Sal´nikova, and V. N. Odinokov*

Institute of Petrochemistry and Catalysis, Russian Academy of Sciences,
141 prosp. Oktyabrya, 450075 Ufa, Russian Federation.
Fax: +7 (347)284 2750. E-mail: ink@anrb.ru, ponedelkina@rambler.ru

Hyaluronic and carboxy hyaluronic acid conjugates with pharmacologically important amines were synthesized and their *in vitro* biodegradability upon the action of testicular hyaluronidase was studied.

Key words: hyaluronic acid, carboxy hyaluronic acid, conjugates, pharmacologically important amines, testicular hyaluronidase, biodegradability.

One of the characteristics of biopolymers for medicobiological purpose is their biodegradability in living organisms, and, depending on the field of application, the biopolymers should possess either increased, or reduced biodegradability upon the action of enzymes.[1] For medicines derived from hyaluronic acid (HA) and used for treatment of arthritis of different etiologies, the reduced biodegradability upon the action of hyaluronidase is required.[2] Enzymic biodegradability of HA can be decreased by its modification[3] with acid dihydrazides and trihydrazides of different structures,[4,5] by methylation with trimethylsilyldiazomethane.[6] Earlier,[7] we have reported on reduced biodegradability of carboxy hyaluronic acid (carboxy-HA) obtained by the selective oxidation of HA with the NaOCl—NaBr—TEMPO system (TEMPO is the 2,2,6,6-tetramethylpiperidinium-1-oxyl).

In order to look for the new derivatives of HA and carboxy-HA with reduced biodegradability with respect to testicular hyaluronidase (HAase), we synthesized conjugates of these polysaccharides with pharmacologically important amines and hydrazides. Such conjugates can be of interest in the development of pharmaceutical drugs or biocompatible materials of prolonged action.

Results and Discussion

The reaction of HA **1** and carboxy-HA **2** with amines **3a—k** and hydrazides **3l,m** in aqueous medium (pH 4.7—4.8, 20—22 °C) in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) furnished conjugates of HA **4a—m** and carboxy-HA **5a—m** (Scheme 1).

The structure of conjugates **4a—m** (see Scheme 1), together with the native units **A**, includes amide units **B**, as well as isoureide units **C** formed due to the addition of EDC to the carboxy groups of HA (see Ref. 8). The ^{1}H NMR spectra of conjugates **4a—k**, in addition to the singlet for the methyl protons of the MeCON groups in the region δ 2.04—2.10, exhibit signals in the region δ 1.50—1.93, which are due to the effect of the magnetic-anisotropy aromatic rings of the arylamide groups on the methyl protons of the neighboring *N*-acetylglucosaminopyranosyl units[8] (Table 1, Scheme 2).

Contribution of the signals intensity in the region δ 1.50—1.93 into the total integral intensity of the signals for the methyl protons did not virtually depend on dilution of the conjugate solutions, from which we drew a conclusion on the action of the anisotropic effect inside the molecular chain of the modified HA. Intensity of these signals were taken into account when content of units **B** and **C** were determined.

The content of units **B** was determined from the proportions of the reduced to one proton total intensities of signals for the aromatic protons (δ 7.0—9.0) and the MeCON methyl protons (δ 1.50—2.10) (see Table 1), the content of units **C** was determined from the proportions of the reduced to one proton intensities of the singlet for the $N(CH_3)_2$ methyl protons in the region δ 3 and the signals for the MeCON methyl protons in the region δ 1.50—2.10. The content of units **A** was determined as a difference between 100% and the total content of units **B** and **C** (see Table 1).

Unlike conjugates of natural HA, conjugates of carboxy-HA **5a—m** possess better solubility in water and lower viscosity, they did not virtually contain isoureide units **C**.

In conjugates **5a—m**, the glucuronic acid units (GlcA) (units **B´**) and/or the glucosaminuronic acid units

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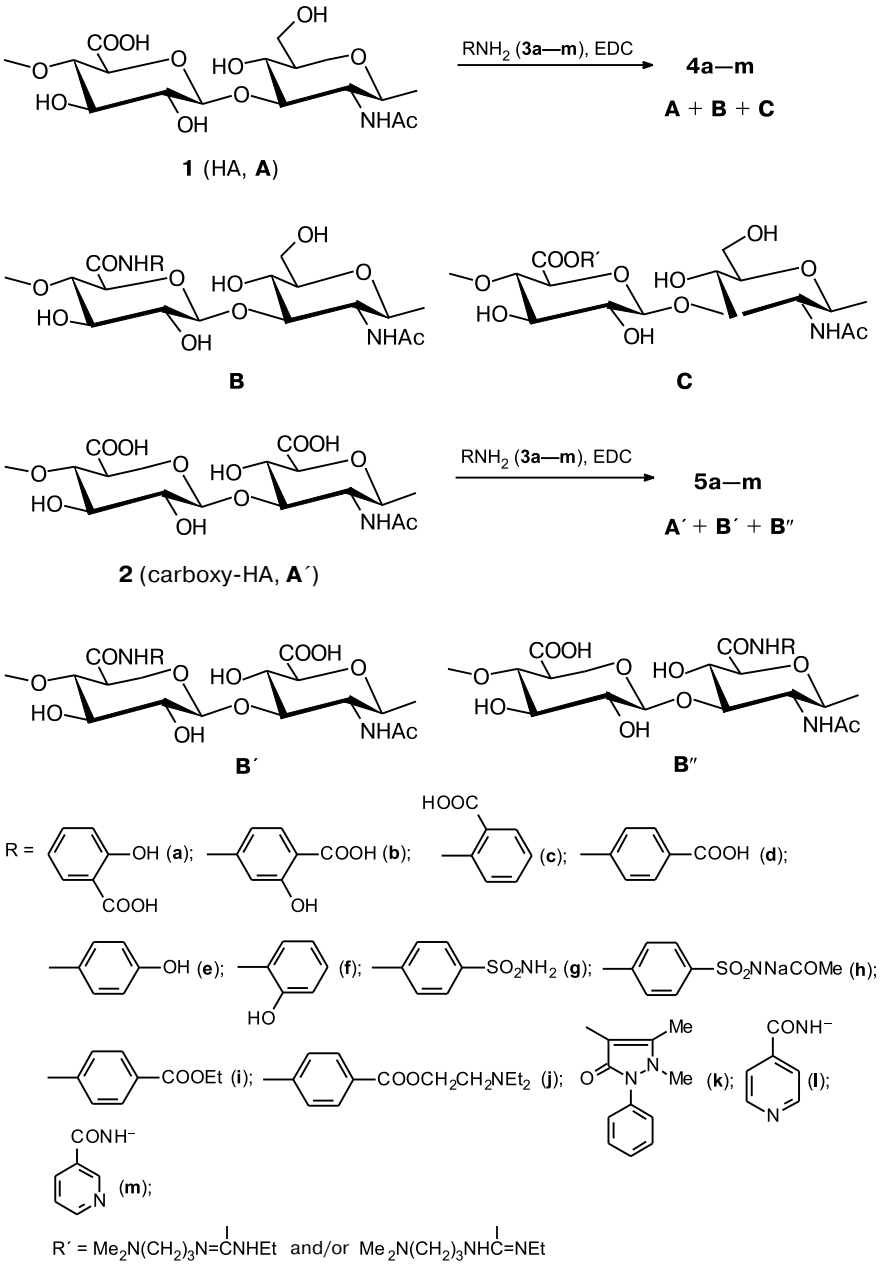
Scheme 1



Scheme 2

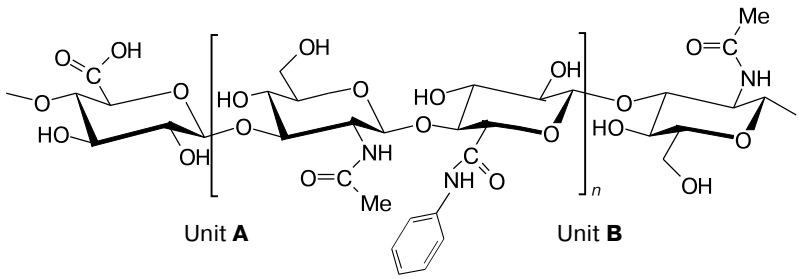


Table 1. Spectroscopic characteristics and content of units* **A**, **B**, and **C** (*N*) in conjugates **4a—m**

Conjugate	^{1}H NMR, δ		*N*		
	MeCON	H_{Ar}	**A**	**B**	**C**
4a	1.65—1.72, 2.10	7.0, 7.7, 8.1	50	40	10
4b	1.56—1.60, 2.04	6.9, 7.5	76	21	3
4c	1.68, 2.10	7.3, 7.6, 8.1, 8.5	30	43	27
4d	1.50—1.70, 2.10	7.8, 8.0	57	18	25
4e	1.58—1.70, 2.08	7.0, 7.5	55	45	0
4f	1.78—1.83, 2.10	7.1, 7.3, 7.6	51	49	0
4g	1.60, 2.08	7.6, 7.9	44	37	19
4h**	1.62, 2.10	7.8, 7.9	70	25	5
4i**	1.62, 2.09	7.8, 8.1	80	15	5
4j**	1.58—1.62, 2.06	7.9, 8.0	42	25	33
4k**	1.93, 2.08	7.5, 7.7	40	57	3
4l	2.05	8.0, 8.9	50	36	14
4m	2.06	7.6-9.0	64	26	10

* Calculated on 100 disaccharide units of HA.

** The ^{1}H NMR spectra of conjugate **4h** exhibit characteristic signal at δ 2.0 (s, CH_3CO); for conjugate **4i**, the signals at δ 1.5 (t, $C\underline{H}_3CH_2O$, J = 7.0 Hz) and 4.5 (m, $CH_3C\underline{H}_2O$); for conjugate **4j**, the signal at δ 1.4 (t, $NCH_2C\underline{H}_3$, J = 7.0 Hz); for conjugate **4k**, the signals at δ 1.9 and 2.4 (s, $C{=}CCH_3$) and 3.4 (s, CH_3N).

(GlcNAcA) (units **B″**) can be modified. The content of the modified units of both types (**B´** + **B″**) was determined from the proportion of the reduced to one proton total intensities of the signals for the aromatic (δ 7.0—9.0) and methyl protons of the acetamide group (δ 2.01—2.03) (Table 2).

The ^{1}H NMR spectra of conjugates **5b**,**f** and **5i—k** exhibit signal for the MeCON group as two singlets of approximately equal intensities at δ 2.01 and 2.03, which indicates that the amide is formed predominantly in the GlcNAcA unit and, consequently, the GlcNAcA is more reactive as compared to GlcA. (The formation of amide at the carboxy group of GlcA, which is remote from the carboxy group of GlcNAcA, does not cause splitting of the signal for the MeCON group.) Since the signal for the methyl protons of GlcNAcA is observed at δ 2.01 in the spectrum of the intact carboxy-HA, the singlet at δ 2.03 was assigned to the modified unit **B″**. Therefore, the con-

Table 2. Spectroscopic characteristics and content of units* **B´** and **B″** (*N*) in conjugates **5a—m**

Conjugate	^{1}H NMR, δ		*N*		
	MeCON	H_{Ar}	**B´**	**B″**	**B´** + **B″**
5a	2.01—2.03	6.96, 7.51, 7.82	—**	—**	23
5b	2.01, 2.03	7.03, 7.18, 7.80	4	26	30
5c	2.01—2.03	7.26, 7.54, 7.93, 8.24	—**	—**	19
5d	2.01—2.03	7.63, 7.98	—**	—**	36
5e	2.01—2.03	6.92, 7.33	—**	—**	25
5f	2.01, 2.03	7.01, 7.20, 7.51	11	24	35
5g	2.01—2.03	7.76, 7.95	—**	—**	30
5h	2.01—2.03	7.77, 7.95	—**	—**	20
5i	2.01, 2.03	7.72, 8.08	8	23	31
5j	2.01, 2.03	7.66, 8.03	3	27	30
5k	2.01, 2.03	7.43, 7.61	8	27	35
5l	2.01—2.03	8.74, 7.82	—**	—**	35
5m	2.01, 2.03	7.6—9.0	5	27	32

* Calculated on 100 monosaccharide units of carboxy-HA. To obtain the content of units **B″** calculated on 100 monosacharide units of carboxy-HA, the result obtained from the ratio of intensity of the singlet at δ 2.03 to the total intensity of the singlets at δ 2.01 and 2.03 (calculated on 100 disacharide units) was divided by 2.

** Units **B´** and **B″** in the ^{1}H NMR spectra of these conjugates are indistinguishable.

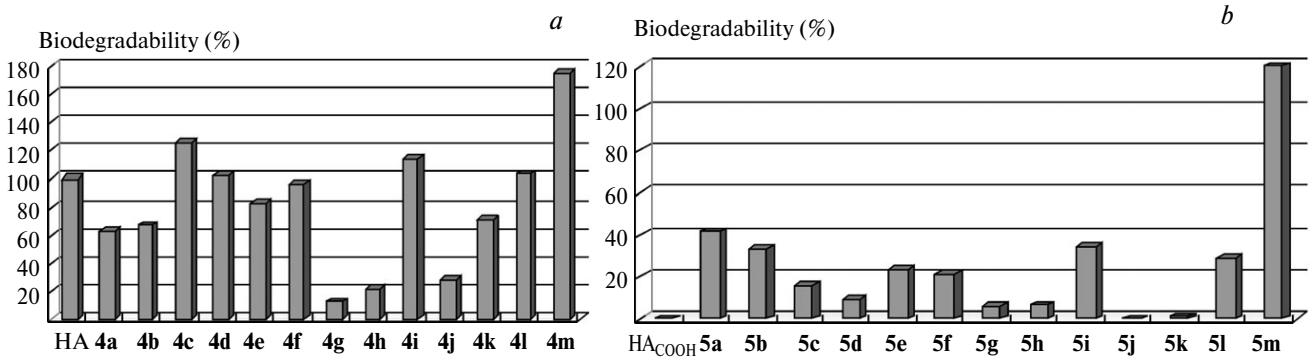


Fig. 1. Relative biodegradability (in percentage to biodegradability of HA) of HA conjugates **4a—m** (*a*) and carboxy-HA conjugates (HA_{COOH}) **5a—m** (*b*).

tent of units **B″** was found from the proportion of intensity of the singlet at δ 2.03 to the total intensity of the singlets at δ 2.01 and 2.03. The content of units **B´** was found as a difference between the total content of the modified units and the content of units **B″** and, as it is seen from the data in Table 2, did not exceed 3—11%.

The signals for the methyl protons of the MeCON group were observed as one broad singlet in the ^{1}H NMR spectra of conjugates **5a**, **5c—e**, **5g**,**h**,**l**,**m** (see Table 2), and units **B´** and **B″** were indistinguishable, however, they can be basically determined as units **B″** because of higher reactivity of GlcNAcA as compared to GlcA.

The shielding magnetic-anisotropy effect of the aromatic ring of the arylamide group of the modified unit on the methyl protons of the acetamide group in the unit GlcNAcA, which is characteristic of the conjugates of natural HA, was observed in the ^{1}H NMR spectra of conjugates only at high conversions of carboxy groups to arylamide ones. For example, in the ^{1}H NMR spectra of conjugates **5e** and **5k** with the content of modified units 81 and 90%, respectively, an additional high-field signal (δ 1.71 for **5e** and 1.82 for **5k**) was observed together with the main signal for the methyl protons of the acetamide group in the region δ 2.01—2.03. Allowance was made for this fact when the conversion was determined by summing up intensities of both signals.

Relative biodegradability *in vitro* (in percent to biodegradability of the native HA) of conjugates of HA **4a—m** and carboxy-HA **5a—m** upon the action of testicular hyaluronidase (HAase) was studied by the known procedure[7] using the Dische reaction[9] for determination of GlcA in the low-molecular-weight products of enzymic cleavage. As it was found in the control experiments, GlcNAcA was not involved into the Dische reaction,[7] uronic amides (or products of their hydrolysis with 87% H_2SO_4, 100 °C) did not interfere with the coloring of the solutions.

Biodegradability of conjugates **4a—m** and **5a—m** depended on both the nature of modified units (Fig. 1) and their content (Fig. 2 and 3). Biodegradability of conjugates **4c** (with anthranilic acid) and **4i** (with anestesine) was higher (by 10—30%) than that of native HA, biodegradability of conjugates **4d** (with *p*-aminobenzoic acid), **4f** (with *o*-aminophenol), and **4l** (with isonicotinic acid hydrazide) was on the level with HA, whereas biodegradability of conjugates **4a** (with 5-aminosalicylic acid), **4b** (with 4-aminosalicylic acid), and **4e** (with *p*-aminophenol) was lower by 20—35% (see Fig. 1, *a*). For conjugates **4h** (with sulfacyl-sodium) and **4j** (with novocaine) with high content of units **C**, biodegradability proved considerably lower (by 70—85%) with respect to that of HA, since the presence of units **C** decreased the solubility of the conjugates in water and, therefore, increased their stability to-

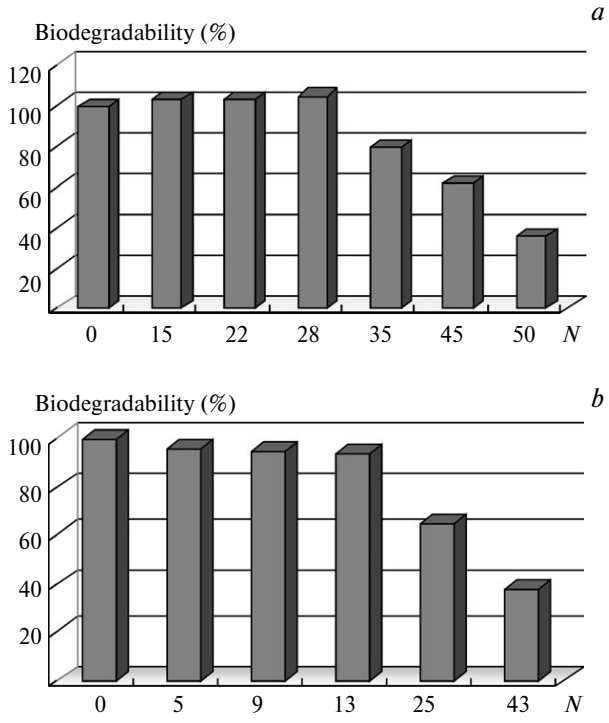


Fig. 2. Relative biodegradability (in percentage to biodegradability of HA) of HA conjugates **4e** (*a*) and **4k** (*b*) depending on the content of the modified units (*N*).

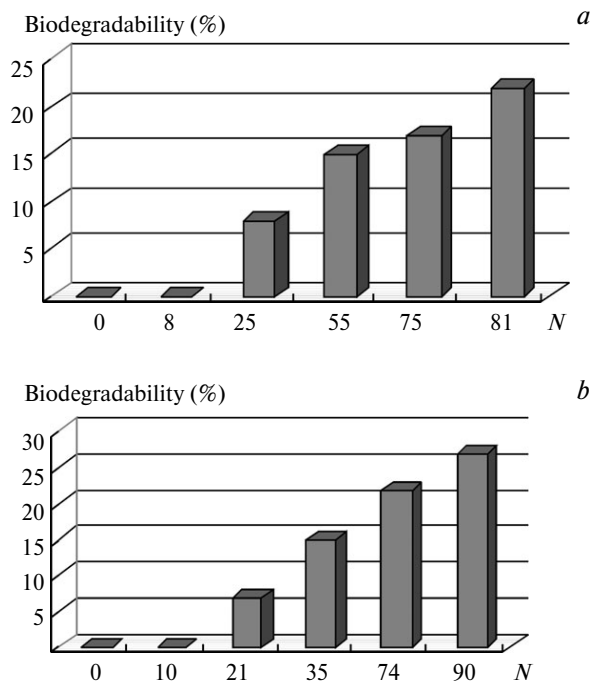


Fig. 3. Relative biodegradability (in percentage to biodegradability of HA) of carboxy-HA conjugates **5e** (*a*) and **5k** (*b*) depending on the content of the modified units (*N*).

ward the action of HAase. It should be noted that conjugate **4m** (with nicotinic acid hydrazide) exceeded HA in biodegradability by 75%. Apparently, this is explained by the fact that the covalently bound nicotinic acid increased activity of HAase, like nicotinic acid itself.[10] Conjugates with increased biodegradability can be useful for burn disease, when high-calorie nourishment of cells and tissue repair are required.

The lower biodegradability of carboxy hyaluronic acid **2** with respect to native HA **1** has been explained earlier.[7] Biodegradability of conjugates of carboxy-HA **5a—l** was significantly lower than that of natural HA, but higher than that of intact carboxy-HA, and also depended on the nature (see Fig. 1, *b*) and content (see Fig. 3) of the modified units. Biodegradability of conjugates upon the action of HAase increased with the increase in the amount of modified units (see Fig. 3, conjugates **5e** and **5k**). Possibly, this is due to the changes in the conformation of the macromolecule: with the increase in the content of substituents, the availability of the polysaccharide (1→4)-glycoside bonds for the cleavage by HAase also increases. Similarly to conjugate **4m**, conjugate **5m** activated the action of HAase and had higher biodegradability as compared to conjugates **5a—l** (Fig. 1, *b*).

Experimental

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVANCE 400 spectrometer (400.13 and 100.58 MHz, respectively) in D_2O. An pH-340 pH-meter was used to control the pH of solutions. 5-Aminosalicylic (**3a**), anthranilic (**3c**), *p*-aminobenzoic (**3d**) acids and *o*-aminophenol (**3e**) (pure for analysis grade), *p*-aminobenzenesulfonamide (streptocide, **3g**), ethyl *p*-aminobenzoate (anestesine, **3i**), and β-diethylaminoethyl *p*-aminobenzoate (novocaine, **3j**) of pharmacopoeia purity were used in the work; *p*-aminobenzenesulfonylacetamide-sodium (sulfacyl-sodium, **3h**) and isonicotinic acid hydrazide (isoniazide, **3l**) were isolated from the aqueous solutions of the corresponding medicines; EDC, 4-aminosalicylic acid (**3b**), *p*-aminophenol (**3f**), 4-amino-2,3-dimethyl-1-phenylpyrazolone-5 (4-aminoantipyrine, **3k**), and nicotinic acid hydrazide (**3m**) were purchased from Aldrich. Testicular hyaluronidase (pharmacopoeia preparation Lidaza) was used without additional purification. Hyaluronic and carboxy hyaluronic acids were obtained according to the procedures given in the works,[7,8] respectively. Citrate buffer (pH 6.3) was prepared from citric acid (30 mmol), Na_2HPO_4 (150 mmol), and NaCl (150 mmol) in H_2O (1000 mL).

Synthesis of conjugates 4a—m and 5a—m (general procedure). Aqueous 0.1 *M* NaOH (0.1 *M* HCl) was added to a mixture of HA **1** (60 mg, 0.15 mmol of COOH groups) or carboxy hyaluronic acid **2** (65.6 mg, 0.30 mmol of COOH groups) and the corresponding amine **3a—m** (0.15—0.60 mmol) in H_2O (15 mL) to pH 4.7—4.8, then EDC (0.11—0.90 mmol) was added with vigorous stirring at 20—22 °C, maintaining pH of the reaction mixture at 4.7—4.8 for 1 h using 0.1 *M* HCl. Then NaOH (0.1 *M*, to pH 7), brine (2—3 mL), and cold MeOH (45 mL) were sequentially added to the mixture. A precipitate was separated by centrifugation, washed with MeOH (3×10 mL), then with Et_2O (3×10 mL), and dried *in vacuo* at temperatures not higher than 60 °C. The yields of conjugates **4a—m** and **5a—m** were 75—80%, white powders with the pink or yellow tint. Characteristic signals in the ^{1}H NMR spectra of conjugates **4a—m** and **5a—m**, important for the determination of the content of modified units, as well as the content of units, are given in Tables 1 and 2.

Determination of relative biodegradability of conjugates 4a—m and 5a—m. A solution of HAase (3 c.u.) in citrate buffer (0.1 mL) was added to a citrate buffer (0.9 mL) containing the corresponding conjugate **4a—m** or **5a—m** (0.01 mmol), the mixture was incubated for 20 h at 37 °C, followed by addition of the MeOH—Et_2O (3 : 1) mixture (4 mL), kept for 1 h at 0 °C, a precipitate was separated by centrifugation. The supernatant was placed into a glass and concentrated, the solid residue was dissolved in an exactly measured volume of water (from 5 to 10 mL), the obtained solution of the low-molecular-weight products of enzymic degradation was analyzed for the content of D-glucuronic acid by the Dische reaction,[9] taken biodegradability of the native HA for the same concentration of HA-ase as 100%. The error of determination was ±5%.

Biodegradability depending on the content of modified units was determined using conjugates **4e**,**k** and **5e**,**k**, which were well soluble in water independent on the content of amide units.

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