Luminescent polyelectrolytes with antiviral activity

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Radical polymerization was used to synthesize and characterize (co)polymers with sodium styrenesulfonate (NaSS), 4-methacryloylamidosalicylic acid (MASA), and N-vinylpyrrolidone, which have a low cytotoxicity and a high antiviral activity against the human respiratory syncytial virus. The interaction of copolymers with Tb 3+ ions was studied. The complexes formed in dilute aqueous solutions at a concentration of MASA units c ≤ 1 • 10−4 mol L −1 demonstrate a strong luminescence. The luminescence intensity is independent of copolymer composition, but increases when the NaSS units are substituted with uncharged N-vinylpyrrolidone units. The obtained Tb 3+ polymer complexes are promising luminescent sensors for the visualization of biological objects interacting with copolymers.

Key words: luminescent metal–polymer complexes, lanthanides, sodium polystyrene-sulfonate, 4-methacryloylamidosalicylic acid, antiviral activity.

In the context of the expected pandemic waves due to the emergence of new mutant strains of the SARS-Cov-2 coronavirus and their coincidence with the seasonal waves of traditional respiratory infections, the development of novel approaches of antiviral therapy is extremely relevant. Therefore, the search for antiviral drugs that are effective and non-toxic for the host cells is an important task. Among the various promising classes of substances, macromolecular compounds are of particular interest to researchers. As early as the end of the 20th century, it was found that some polyelectrolytes demonstrate antiviral activity.1—5 Antiviral activity was observed in various sulfonated polysaccharides,2—4 in polyacrylic and polymethacrylic acids, as well as in copolymers of maleic anhydride, vinylamine, etc.5 The activity of these polyelectrolytes is a result of the ability of their macromolecules to block the stage of virion adhesion on cells.6,7 In modern conditions, it is advisable to search for macromolecules that stimulate the development of a nonspecific resistance of the body to external infections. In this regard, the synthesis of a series of new polyelectrolytes having different chemical structures and varying molecular weights, as well as the determination of a relationship between the structure and the level of biological activity, is of particular importance. When developing these polymers, it is necessary to study the processes of their interaction with viruses and cells, pharmacokinetics, etc. This requires the synthesis of macromolecules with luminescent labels that allow the visualization of the localization of these objects in tissues and cells. The use of Eu 3+, Tb 3+, Sm 3+ ions, which luminesce in the visible region of the spectrum, as labels is promising.8 The binding of these ions with the target macromolecules is ensured by the introduction of special chelating groups into their structure, of which aromatic carboxylates, in particular, p-aminosalicylic acids (PASA), are of interest. p-Aminosalicylic acid contains an aromatic nucleus with carboxyl and hydroxyl groups, can coordinate polyvalent metal ions and provide the transfer of excitation energy from the triplet level of the ligand to the resonant level of lanthanide ions.9—11
Therefore, it is an effective sensitizer of their luminescence.

The goal of this work is to synthesize anionic polyelectrolytes, namely copolymers of methacryloyl-amidosalicylic acid (MASA), which acts as a chelate site, with sodium styrenesulfonate (NaSS) and N-vinylpyrrolidone (VP), to evaluate their antiviral activity and cytotoxicity, as well as to study the photophysical properties and conditions of the formation of their luminescent complexes with Tb$^{3+}$.

**Experimental**

The following reagents were used in the work: VP (Aldrich, cat. No. V3409) purified by vacuum distillation (b.p. 69 °C (3 Torr), $n_D^{20} = 1.5120$); NaSS (Aldrich, cat. No. 32.859-6); AIBN (Porofof ChKhZ257, 99%, LDKhim Ltd.); PASA (99%, Acros Organics, cat. No. 104621000); methacryloyl chloride (90%, Aldrich, cat. No. 32.037-4); Et$_2$O (analytical grade, Kuzbasorgkhim Ltd.); NaOH (reagent grade, Lenreaktiv Inc.); DMSO (reagent grade, Vekton); PriOH (90%, Aldrich, cat. No. V3409); Me$_2$CO (reagent grade, Vekton); Na$_2$CO$_3$ (reagent grade, Yuventa); MEM culture medium (Minimal essential media) in Dulbecco modification (DMEM) with 1-glutamine and glucose (Reg. Cert. FSR 2008/03103, Biolot); Tween 20 (RFE, USP-NF, BP, Ph.Eur., for biochemistry, Biolot); phosphate-buffered saline (tablets, Biolot); tetramethylbenzidine (for biochemistry, no less than 98% (titer), AppliChem); penicillin-streptomycin (Gibo, USA); fetal bovine serum (FBS, Gibo, USA) and bovine serum albumin (BSA, KhIMEKS Ltd.).

IR spectra were recorded on a Vertex-70 Fourier-transform spectrophotometer (Bruker) with an ATR unit (Pike), $^1$H and $^{13}$C NMR spectra were recorded on a Bruker AVANCE-400 instrument in DMSO-d$_6$. Chemical shifts for $^1$H are given relative to the solvent residual proton signal ($\delta$ 2.50), chemical shifts for $^{13}$C are given relative to the DMSO-d$_6$ signal ($\delta_{C} 39.53$).

Absorption spectra of solutions of (co)polymers were recorded on a SF-256 UVI spectrophotometer (LOMO Fotonika Ltd., Russia), photoluminescence excitation and emission spectra of solutions were recorded on a LS-100 spectrofluorimeter (PTI, Canada). The widths of the entrance and exit slits of the monochromator were equal to 4 nm. The measurements were carried out in a thermostated cell at 25 °C in a 1-cm quartz cuvette. The solution luminescence intensity measurement error did not exceed 3%.

Synthesis of MASA was carried out by acylation of PASA with methacryloyl chloride in an aqueous alkaline medium in accordance with the known procedure,$^{12}$ then it was purified by recrystallization from an aqueous-alcoholic medium, m.p. 208 °C (cf. Ref. 12; m.p. 206—207 °C). The structure of the monomer was confirmed by spectroscopic methods. $^1$H NMR, $\delta$: 13.65 (COOH); 11.32 (Ar—OH); 10.00 (CONH); 7.71 (d, 1 H, H(9), $J = 8.7$ Hz); 7.43 (d, 1 H, H(6), $J = 1.5$ Hz); 7.23 (dd, 1 H, H(10), $J_1 = 8.7$ Hz, $J_2 = 1.5$ Hz); 5.82, 5.57 (both s, 1 H each, H$_2$C=C); 1.96 (s, 3 H, Me). $^{13}$C NMR, $\delta$: 16.65 (C(3)); 106.75 (C(8)); 111.02 (C(10)); 120.78 (C(1)); 130.79 (C(9)); 140.16 (C(3)); 145.58 (C(5)); 161.98 (C(7)); 167.39 (C(4)); 171.67 (C(11)). The signals in the NMR spectra were assigned based on 2D COSY, HSQC, and HMBC spectra.

The complexation of Tb$^{3+}$ ions with (co)polymers was studied in aqueous solutions. A weighed portion of the (co)polymer was dissolved in distilled water and the pH of...
the solution was adjusted to 7.5—8.0 by adding 0.1 M NaOH. The series of solutions for measuring luminescence was prepared with the same concentration of MASA units (4 × 10⁻⁵ mol L⁻¹) and a variable concentration of TbCl₃. The solutions were allowed to stand for 60 min before measuring the luminescence intensity. The solution of BSA was prepared at pH 7.7 by adding 0.1 M NaOH.

Cytotoxicity and antiviral activity were determined using the HEp-2 cell culture, the epidermoid carcinoma of the human larynx obtained from the collection of cell cultures of the Laboratory of Cell Cultures of the A. A. Smorodintsev Research Institute of Influenza. In this work, we used the human respiratory syncytial virus, strain A2, which was obtained on April 1, 2018 from the Laboratory of Biotechnology of Diagnostic Preparations of the A. A. Smorodintsev Research Institute of Influenza of the Ministry of Health of the Russian Federation, then accumulated on the HEp-2 cell culture and stored at –80 °C. The DMEM medium with an antibiotic (penicillin—streptomycin), FBS, and l-glutamine served as the maintenance medium.

The cytotoxicity (CC₅₀) of polymers was assessed taking into account cell viability using a microtetrazolium test. Antiviral activity was determined by the enzyme immunoassay (EIA) method. A series of 3-fold dilutions of polymers was prepared, starting from 1/2 CC₅₀, then applied to the HEp-2 cell culture in double concentration (100 μL per well), immediately followed by the addition of the virus (100 μL in a series of 10-fold dilutions), then undergoing a repeated incubation for 1 h at 37 °C in the presence of CO₂ (5%). Then the virus was washed off, the preparations were applied at the same concentration, followed by incubation for 6 days at 37 °C in the presence of CO₂ (5%).

To carry out ELISA, the cell culture was fixed with cold 80% acetone for 15 min, washed with the phosphate-buffered saline solution (pH 7.4; NaCl, 8.00 g; KCl, 0.20 g; Na₂HPO₄, 1.44 g; KH₂PO₄, 0.24 g; distilled water, 800 mL), then distilled water was added to reach a volume of 1 L) with the addition of Tween 20 to 0.05%, a solution of primary mouse antibodies to the respiratory syncytial virus F protein was applied to the culture, followed by incubation for 2 h at room temperature and with continuous stirring.

Next, the cells were washed with a buffer, secondary anti-mouse antibodies were applied, followed by repeated incubation for 2 h with continuous stirring, then the antibodies were washed off and the substrate-chromogenic mixture with tetramethylbenzidine was applied. After 5 min, the reaction was stopped using 0.1 M sulfuric acid, and the optical density of the solution at λₘₐₓ 450 nm was determined. Wells, in which the absorbance exceeded that of the wells with control cells by a factor of two or more, were considered to be contaminated. The virus titer was calculated according to the Reed and Muench method.

The criterion for evaluating antiviral activity is a statistically significant decrease of the titer of the virus in cells when applying the polymer compared to the control. Based on these results, we calculated IC₅₀, the effective drug concentration at which the virus titer is reduced by 50%.

To assess the prospects of the (co)polymer as an antiviral agent against the respiratory syncytial virus, we used the selective index (SI), which is equal to the ratio of CC₅₀ to IC₅₀. Compounds with SI values of more than 8 were considered promising.

### Results and Discussion

The synthesis conditions and characteristics of the obtained homopolymer poly-NaSS (1) and copolymers [NaSS]₁₉—[MASA]₇ (2a), [NaSS]₁₆₄—[MASA]₁₆ (2b), [VP]₉₃—[MASA]₇ (3a), [VP]₇₄—[MASA]₂₆ (3b), as well as their biological properties are given in Table 1.

For polymers 1—3, cytotoxicity was determined and in vitro antiviral activity was tested in relation to the human respiratory syncytial virus A2. All the studied compounds demonstrated a high antiviral activity against the respiratory syncytial virus A2. The cytotoxicity of the drugs was similar and varied within 163—325 μg mL⁻¹.

In copolymers 2, Tb³⁺ ions can interact with both anions of MASA and NaSS. The complexation ability of styrenesulfonates is reduced compared to that of salicylate anions, because they are strong acid anions.

The absorption spectra of the solution of homopolymer 1 contain a band at λₘₐₓ = 262 nm, while in

### Table 1. Synthesis conditions and characteristics of the obtained homo- and copolymers 1—3 and their biological properties

| Compound | Starting mixture composition (mol.%) | Solvent (wt.%) | Yield (wt.%) | Copolymer composition (mol.%) | [η]²⁵/²⁵/dL g⁻¹ | CC₅₀ (μg mL⁻¹) | IC₅₀ (μg mL⁻¹) | SI |
|----------|-------------------------------------|----------------|--------------|-----------------------------|----------------|----------------|---------------|----|
| 1        | NaSS 100  MASA 0  VP 0             | DMF + PrOH 90  | 100          | NaSS 100  MASA 0  VP 0      | 0.63           | 163            | 3.5           | 47 |
| 2a       | NaSS 85  MASA 15  VP 0              | H₂O + DMF 84  | 93           | NaSS 84  MASA 16  VP 0     | 0.41           | 325            | 3.4           | 96 |
| 2b       | NaSS 70  MASA 30  VP 0              | H₂O + DMF 80  | 84           | NaSS 84  MASA 16  VP 0     | 1.34           | 191            | 1.3           | 147|
| 3a       | NaSS 0  MASA 10  VP 90              | EtOH 66       | 0            | NaSS 0  MASA 7  VP 93     | 1.5            | 182            | —             | —  |
| 3b       | NaSS 0  MASA 20  VP 80              | EtOH 60       | 26           | NaSS 26  MASA 7  VP 74    | 0.25           | 193            | 6             | 34 |

* Characteristic viscosity in 0.1 M NaCl.

Drug dose at which the reproduction of the virus is halved.
the spectrum of copolymer 2a this band shifts to 265 nm and a band at \( \lambda_{\text{max}} = 305 \) nm appears, originating from the electronic \( n-\pi^* \)-transitions in the carboxylate anion (Fig. 1). The addition of \( \text{TbCl}_3 \) to the solution 2a does not change the absorption spectrum.

Figure 2 shows the spectra of luminescence excitation of \( \text{Tb}^{3+} \) in solutions of copolymers 2a and 2b. As can be seen from Fig. 2, the shape of the excitation spectrum of the solution of \( \text{Tb}^{3+}/2 \) differs considerably from the shape of its absorption spectrum (see Fig. 1). This is due to the fact that the intensity ratio of the bands depends not only on the molar absorption coefficients of the ligand and the lanthanide ion, but also on the efficiency of luminescence sensitization, determined by the composition of the copolymer and the structure of the inner sphere of the lanthanide complex, that is, the number and ratio of \( \text{COO}^- \) groups bound by the \( \text{Tb}^{3+} \) ion and water molecules. It also follows from Fig. 2 that an increase in the content of MASA units within the copolymer from 7 to 16 mol.% has virtually no effect on the shape of the excitation spectra.

The photoluminescence spectra of solutions of \( \text{Tb}^{3+}/2a \) and \( \text{Tb}^{3+}/2b \) (Fig. 3) contain bands characteristic of the \( \text{Tb}^{3+} \) ion (489 nm \( (\text{3D}_4 \rightarrow \text{7F}_6) \), 544 nm \( (\text{3D}_4 \rightarrow \text{7F}_5) \), 582 nm \( (\text{3D}_4 \rightarrow \text{7F}_4) \)), and the ligand luminescence band at \( \lambda = 402 \) nm is retained, its intensity decreasing with increasing concentration of the \( \text{Tb}^{3+} \) ions.

Figure 4 shows the dependencies of the luminescence intensity of \( \text{Tb}^{3+} \) ions in the band at \( \lambda_{\text{max}} = 544 \) nm
(I_{544}) on their concentration in solutions 2a and 2b. As can be seen in Fig. 4, an increase in the content of MASA units within the copolymer has almost no effect on the Tb^{3+} luminescence intensity.

For comparison, Fig. 4 shows a similar dependence for a solution of Tb^{3+} and copolymer VP-MASA. In the macromolecules 3a, Tb^{3+} ions interact only with salicylate anions, and, due to their low content, a complex with the maximum possible number (three) of COO− coordinated groups is formed. When studying solutions of copolymers with NaSS, it was found that for the same content of Tb^{3+}, the I_{544} of the solution of Tb^{3+}/2 is considerably lower than that of the solution of Tb^{3+}/3a. This indicates that the styrenesulfonate anion, despite its low complexation ability, binds the Tb^{3+} ions due to the high concentration of its units in the copolymer (the concentration of styrenesulfonate units is 1.5 orders of magnitude higher than the concentration of MASA units), but does not form luminescent complexes. In addition, the possibility of the formation of complexes in which the Tb^{3+} ions are simultaneously bound to salicylate and styrenesulfonate anions is not ruled out, which also leads to a decrease of luminescence intensity. Fragments of the expected structures (A—C) of the complex of Tb^{3+}/2 are shown below.

\[ \begin{align*}
\text{A} & : \quad \text{CH}_2-\text{CH}_n-\text{C}=\text{O} \quad \text{C}_n \quad \text{CH}_2-\text{C}=\text{O} \\
\text{B} & : \quad \text{CH}_2-\text{CH}_n-\text{C}=\text{O} \quad \text{C}_n \quad \text{CH}_2-\text{CH}_m \quad \text{NH} \quad \text{O}-\text{Tb}-\text{O}\cdot\text{C}=\text{O} \\
\text{C} & : \quad \text{CH}_2-\text{CH}_m \quad \text{CH}_2-\text{CH}_m \quad \text{O}_2\text{S} \quad \text{O}-\text{Tb}-\text{O}\cdot\text{SO}_2
\end{align*} \]

To assess the prospects of using the obtained Tb^{3+} complexes as luminescent probes in medicine and biology, it is necessary to study their photophysical properties and stability under conditions approaching physiological, when competition with proteins for Tb^{3+} binding is possible. Thus, the photoluminescence of the complexes was studied in the presence of a protein. BSA was chosen as the model protein.17,18 Figure 5 shows the photoluminescence spectra of the solution of Tb^{3+}/2a with added BSA. The I_{544} of the solution of Tb^{3+}/2a in the presence of BSA varies slightly up to [BSA] = 6 \cdot 10^{-5} mol L^{-1} (4 mg mL^{-1}) and decreases only by a factor of 2 at concentrations approaching the concentrations of albumin in blood plasma (20—30 mg mL^{-1}). Bigger changes are observed for the ligand luminescence band at λ = 402 nm. At a low content of BSA in the solution, I_{402} increases by a factor of almost 2, which indicates a decrease in the efficiency of transfer of electronic excitation energy from the triplet level of the ligand to the resonant level of the Tb^{3+} ion, while the solution I_{544} increases. When the BSA content is further increased, I_{402} does not change, while I_{544} decreases.

A study of the intrinsic luminescence of a BSA solution ([BSA] = 1.1 mg mL^{-1}) showed that the addition of an aqueous solution of Tb^{3+} does not affect the luminescence of BSA, while the addition of a solution of
copolymer 2a (ratio BSA molecule : copolymer unit is equal to 1 : 7 or 1 : 110) leads to a decrease of the intrinsic luminescence of BSA by a factor of 1.7 or 4.8, respectively. The obtained result indicates the interaction of BSA with 2a and the formation of a protein—copolymers complex. It can be assumed that the interaction of BSA with Tb3+ ions. The complexes demonstrate strong intrinsic luminescence of BSA by a factor of 2 at BSA concentrations close to physiological.

In conclusion, homo- and copolymers of NaSS and MASA, which demonstrate antiviral activity toward respiratory syncytial virus A2, were synthesized in this work. The obtained copolymers are promising substrates for the development of drugs, since their selective index is more than an order of magnitude higher than the minimum acceptable value (SImin > 8). Biovisualization of the synthesized copolymers in tissues and cells can be accomplished using their complexes with Tb3+ ions. The complexes demonstrate strong luminescence in dilute aqueous solutions (concentrations [MASA] = 4·10−5 mol L−1 and [Tb3+] = 4·10−5 mol L−1). The luminescence of the complex of Tb3+/2 in BSA solution remains strong, decreasing by a factor of 2 at BSA concentrations close to physiological.

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