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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Encapsulation of Neurotoxins, Blockers of Nicotinic Acetylcholine Receptors, in Nanomaterials Based on Sulfated Polysaccharides

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Abstract—Three-finger snake neurotoxins are selective antagonists of some nicotinic acetylcholine receptor subtypes and are widely used to study these receptors. The peptide neurotoxin azemiopsin, recently isolated from the venom of *Azemipos feae*, is a selective blocker of muscle-type nicotinic acetylcholine receptor. In order to reduce their toxicity and increase resistance under physiological conditions, we have encapsulated these toxins into nanomaterials. The study of nanomaterials after interaction with neurotoxins by the methods of transmission electron microscopy and dynamic light scattering revealed an increase in the size of nanoparticles, which indicates the inclusion of neurotoxins in nanomaterials.

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Nicotinic acetylcholine receptors (nAChRs) are involved in the nerve impulse transmission through synapses and are activated by acetylcholine. nAChRs are found in chemical synapses in the central and peripheral nervous system, neuromuscular junctions, and in the epithelial cells of many animal species. There are a number of peptide protein compounds that selectively interact with nAChR. For example, snake α-neurotoxoins and α-conotoxins, which are antagonists of nAChRs, have long been known [1]. We recently isolated the peptide neurotoxin azemiopsin from the viper *Azemipos feae* venom, which is a selective blocker of the muscle nAChR [2]. nAChRs are involved in many physiological processes, and, in some cases, the regulation of their activity by exogenous ligands, including toxins, is required. For example, we showed that azemiopsin has good prospects for use as a local muscle relaxant [3]. However, there are disadvantages of neurotoxins that prevent their use in practice, the main of which is their high toxicity. Furthermore, the linear peptide azemiopsin may be insufficiently stable under physiological conditions. A possible way to overcome these disadvantages may be the inclusion of neurotoxins in nanomaterials. The available data indicate that that the inclusion of proteins and/or peptides in nanomaterials significantly increases their resistance to degradation under physiological conditions [4]. On the basis of nanomaterials, systems for delivery into the body of peptide and protein drugs (in particular, insulin) through the gastrointestinal tract are being developed [5]. Encapsulation of bee venom in biodegradable nanoparticles based on poly(D,L-lactide-co-glycolide) provided a more long-term effect of the venom while retaining a comparable therapeutic effect [6]. Taking into account these data, we have prepared neurotoxins encapsulated in nanomaterials.

Thermosensitive nanogel consisting of heparin and pluronic 123 (Hep–P123) and the complex of the dendrimer polyamidoamine (PAMAM) of G3.0 generation with the sulfated polysaccharide fucoidan (G3.0–Fu) were used as nanomaterials for encapsulation. Hep–P123 was synthesized as described in [7]. Briefly, to encapsulate toxins, 25 mg of Hep–P123 was dissolved in 5 mL of water, and the resulting solution was supplemented with 2.5 mg of α-cobratoxin (CTX) or azemiopsin (Az) in 0.1 mL of water. After 20 min of incubation, 2.5 mL of the mixture was frozen and lyophilized. The remaining 2.6 mL of the mixture was incubated for 24 h and then frozen and lyophilized. To prepare the nanomaterial based on the dendrimer and fucoidan, 25 mg of fucoidan was dissolved in 2.5 mL of water, the resulting solution was supplemented with 2.5 mg of CTX or Az in 0.1 mg of water. The mixture was stirred for 15 min and mixed with 25 mg of the dendrimer in 2.5 mL of water. After 10-min incubation, the mixture was frozen and lyophilized.

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Table 1. Characteristics of nanomaterials G3.0-Fu and Hep-P123, containing neurotoxins.

Sample	zeta potential, mV	Particle size (nm) according to	
		dynamic light scattering	transmission electron microscopy
G3.0–Fu	–34.5 ± 0.56	70.23 ± 2.61	32.99 ± 4.78
G3.0–Fu–CTX	–6.67 ± 0.51	205.17 ± 2.81	82.66 ± 18.3
G3.0–Fu–Az	–3.33 ± 0.35	204.93 ± 2.94	52.97 ± 5.47
Hep–P123	–60.52 ± 1.73	145.23 ± 6.42	69.4 ± 29.7
Hep–P123–CTX	–45.03 ± 1.46	168.03 ± 1.27	111.74 ± 53.46
Hep–P123–Az	–36.03 ± 1.70	162.80 ± 2.07	71.32 ± 23.66

The obtained materials were analyzed by transmission electron microscopy and dynamic light scattering, and their zeta potential was also measured.

It should be noted that the molecule of the PAMAM dendrimer G3.0 has interior cavities, in which other compounds, including drugs, can be trapped [8]. However, since the dendrimer is highly toxic, its use for biomedical purposes is problematic. The zeta potential of PAMAM G3.0 is 39.3 mV. Due to the high positive zeta potential and strongly basic properties of dendrimers, they can exhibit cytotoxicity, the severity of which depends on the nature and generation of the dendrimer. PAMAM G3.0 does not have a high cytotoxicity. For example, this dendrimer had no toxic effect on MCF-7 and A549 cells up to a concentration of 72 μM [9]. Nevertheless, in this study, to change the surface charge, PAMAM G3.0 was modified using fucoidan, which has a zeta potential of –90.6 mV. Fucoidan is found in some species of brown and brown algae; it exhibits anticoagulant, immunomodulating, antitumor, and antiviral activity and is used as a food additive. The molecular weight of fucoidans varies in a wide range. The weight of the sample used in this study, according to the manufacturer's specifications (F5631, Merck), was 20–200 kDa. The elongated fucoidan molecule substantially exceeds the dendrimer molecule in size. It can be assumed that, after the formation of a complex with the dendrimer, part of the carbohydrate chains of fucoidan containing sulfate groups will be exposed to the solution, forming cavities, which, similarly to the cavities in the dendrimer, can trap other compounds. These cavities are an ideal place for encapsulating highly basic toxins.

As for Hep–P123, its component pluronic P123 is an amphiphilic compound with a low hydrophilic–lipophilic balance value (HLB = 8) and with a very high polypropylene oxide (PPO)/polyethylene oxide (PEO) ratio. As a result, it can include hydrophobic drugs into the cavity formed by the polymer chain. To supplement this ability by the ability to trap basic compounds, we attached P123 to the acidic proteoglycan heparin and obtained the strongly acidic Hep–P123. This conjugation significantly expands the application of the obtained nanogel for encapsulation.

The results of measuring the zeta potential of the studied nanomaterials using the HORIBA SZ-100 analyzer are summarized in Table 1. These data show that G3.0–Fu has the potential –34.5 mV; i.e., fucoidan (potential –90.6 mV) neutralizes the strongly positive potential of the dendrimer (39.3 mV) and thus, presumably, reduce its cytotoxicity. CTX and Az molecules are strongly basic, and their inclusion in G3.0–Fu leads to a marked decrease in the negative potential. The encapsulation of toxins causes a shift in the potential in the positive direction from –34.5 mV for G3.0–Fu to –6.67 and –3.33 mV for G3.0–Fu–CTX and G3.0–Fu–Az, respectively (Table 1).

The thermosensitive nanogel Hep–P123, due to the sulfated proteoglycan heparin, has a negative zeta potential –60.52 mV. As in the case of G3.0–Fu, the encapsulation of the toxins shifted the potential into the positive direction from –60.52 mV for Hep–P123 to –45.03 and –36.03 mV for Hep–P123–CTX and Hep–P123–Az, respectively (Table 1). The observed changes depend on the capacity of the toxin and on the amount of the toxin loaded in the nanomaterial. A change in the potential also means that the toxin is included in the nanomaterial not only by passive capture in the cavities but also by means of electrostatic interaction between the negatively charged shell of the nanomaterial and the and positively charged toxin. This electrostatic interaction helps the toxin to remain within the shell, which may reduce the effect of the physiological environment on the toxins and prolong their action.

The morphology and size of the nanoparticles were examined by transmission electron microscopy (TEM) with a JEM-1400 JEOL electron microscope and by dynamic light scattering (DLS) with a HORIBA SZ-100 nanoparticle size analyzer. We found that the nature of the toxin (molecular mass and charge) affects the structure of the nanomaterial.

According to TEM data, the starting material G3.0–Fu before the encapsulation of toxins had a nanoparticle size of approximately 33 nm (Table 1). It should be noted that the material was sufficiently homogenous, the difference in particle size was small, and the nanoparticles were fairly uniform. However, the G3.0–Fu nanoparticles were not perfectly spheri-

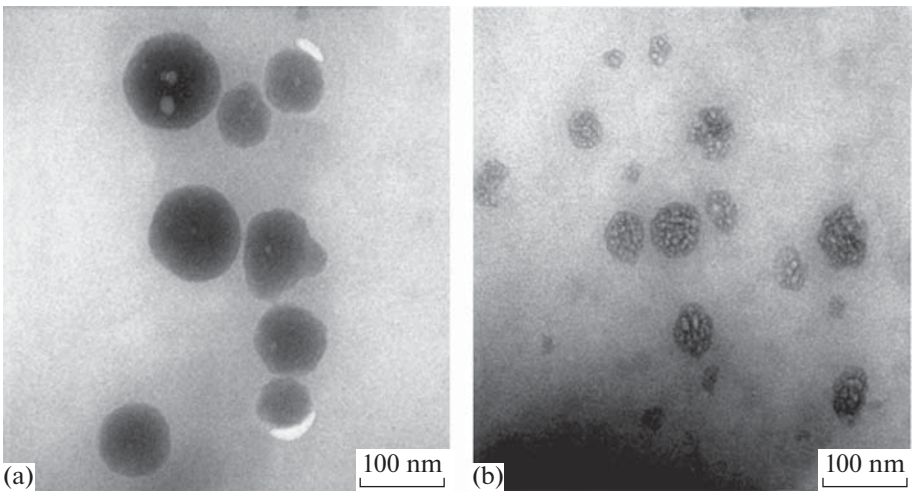

Fig. 1. Electron microscope image of the G3–Fu nanoparticles with encapsulated CTX (a) and Az (b).

cal. The absence of spherical shape can be explained by the fact that this nanomaterial is formed mainly due to the electrostatic interaction between the oppositely charged polyelectrolytes.

Encapsulation of toxins in nanomaterials leads to an increase in size according to both TEM and DLS data (Table 1). As can be seen from the table, the size of nanoparticles determined by DLS substantially exceeds the value determined by TEM. This difference can be explained by the hydrophilic nature of the polysaccharides forming the nanomaterials, which are highly hydrated in water. Hydration results in swelling and increase in the apparent size of nanoparticles determined by DLS. After encapsulation, the size of G3.0–Fu nanoparticle, measured by TEM, increased from 32.99 to 82.66 nm (by 150%) and 52.97 nm (60%) of CTX and Az, respectively (Table 1, Fig. 1). Measurements by DLS showed an increase in the size of nanoparticles from 70.23 to 205.17 nm and 204.93 nm for CTX and Az, respectively (Table 1). The size of nanoparticles after encapsulation of the toxins increase with increasing molecular mass of the toxins, which is 2540 and 7821 Da for Az and the CTX, respectively. As shown by the presented TEM data (Table 1, Fig. 1), the size of nanoparticles with CTX was larger by 29.7 nm (56%) than the size of nanoparticles with Az. Interestingly, according to DLS data, the sizes of both nanomaterials are very similar (Table 1), which is apparently due to the influence of the environment, including hydration. It should be noted that nanoparticles G3.0–Fu–CTX and G3.0–Fu–Az are more uniform in size and have a more spherical shape (Fig. 1) than G3.0–Fu.

The analysis of the size and morphological structure of Hep–P123 nanoparticles showed that, before the encapsulation of the toxins, the size of nanoparticles was 69.4 nm according to TEM data and 145.2 nm according to DLS data (Table 1). The results of TEM showed that, after the encapsulation of Az, the size of Hep–P123 particles was almost unchanged (71.32 nm, 3% increase). However, when the larger CTX molecule was encapsulated, the particle size greatly increased to 111.74 nm (61%) (Table 1, Fig. 2). The presented data (Table 1) show that the size of the nanoparticles with CTX was greater by 40.3 nm (57%) than the size of the nanoparticles with Az. The measurements by DLS showed a slight increase in the size of nanoparticles from 145.23 nm to 168.03 and 162.80 nm for CTX and Az, respectively (Table 1). That is, according to DLS data, the sizes of the nanoparticles with the encapsulated toxins also increased with increasing molecular mass of the toxins. The differences in the sizes revealed by DLS were not so significant (Table 1), which also can be explained by the influence of the external environment. The results of the study of the nanomaterials based on Hep–P123 showed that, in this case, the nanoparticles had a regular spherical shape (Fig. 2). According to TEM data, the size of the nanoparticles varied in the range from 22 to 100 nm; i.e., they were more heterogeneous in size than in the case of G3.0–Fu. This heterogeneity is a characteristic property of Hep–P123 (Fig. 2A) [7], which can be explained by the different degrees of hydrophobicity of the surface of nanoparticles or by the different number of P123 groups attached to heparin. In addition, the Hep–P123 nanoparticles in aqueous solution tend to aggregate in a cloud (in a group). This phenomenon can be explained by the low HLB value of P123 molecules as well as by the high PPO/PEO ratio, which leads to the presence of a large hydrophobic portion in the Hep–P123 particles. To reduce the surface tension, the hydrophobic portions may stick together, thereby displacing the aqueous layer.

It should be noted that, previously, the neurotoxin from the *Naja naja atra* cobra venom was encapsulated

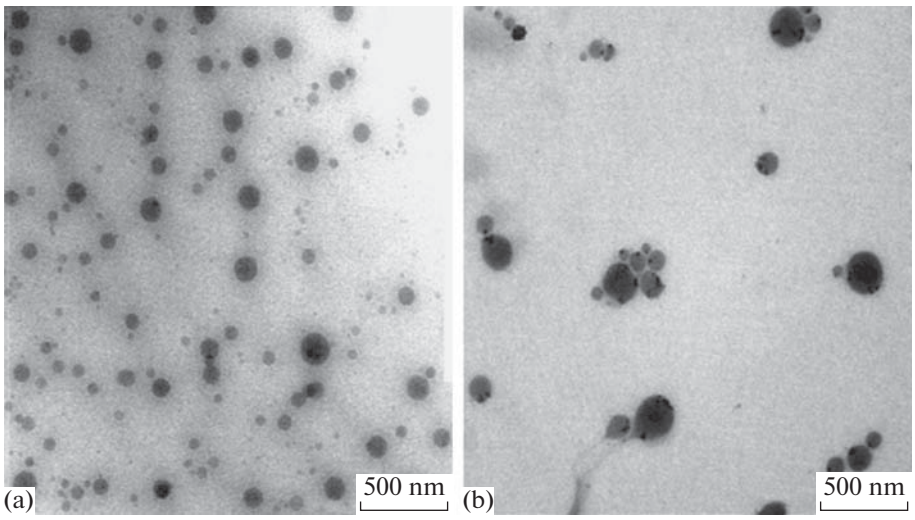

Fig. 2. Electron microscope image of the Hep–P123 nanoparticles (a) and the Hep–P123 nanoparticles with encapsulated CTX (b).

in liposomes based on lecithin and cholesterol [10]. In our study, two neurotoxin—CTX and Az—were encapsulated in two different nanomaterials, G3.0-Fu and Hep-P123, containing sulfated polysaccharides and having different characteristics. The presence of negatively charged polysaccharides in the nanomaterials used by us contributes to a better retention of neurotoxins. G3.0–Fu comprises Fu, which has a high biocompatibility and a high negative charge, whereas the G3.0 dendrimer has cavities inside the molecule and can be used to deliver drugs into cells. Hep–P123 is a thermosensitive nanogel, which can be used to deliver compounds exhibiting hydrophobic properties, which is often observed in the anticancer drugs. Both of these nanomaterials have different physicochemical himichesikmi characteristics, which is reflected in the results of their structural morphological analysis by TEM and DLS, performed in this work.

Thus, the data presented in this study suggest that we for the first time encapsulated snake venom neurotoxins in nanomaterials containing sulfated polysaccharides.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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