An influence of complexes of therapeutic antisense oligodeoxynucleotides with cationic polymers on cell respiration

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Antisense-DNA technologies are new strategy for the treatment of prion infections. This strategy requires prolonged administrations of the drugs, which are likely to alter cell redox processes. **Aim.** The evaluation of cell survival and intensity of oxidative processes *in vitro* under the influence of antisense-oligodeoxynucleotides (asODNs) as cell prion inhibitors (PrP⁰) complexed with cationic polyelectrolyte. **Methods.** Free diffusion in agarose gel, study of cytotoxic action on model cells (bull semen), polarography and potentiometric measurement of oxygen uptake, statistical analysis. **Results.** Poly(dimethylaminoethyl methacrylate)-based surfactants form complexes with asODNs. Polyethylene glycol containing surfactants increase oxygen uptake by cells: by 18 % (VI), by 37 % (IV) and 2.6-fold for V. An addition of the IV-asODNs complex into [the] cell medium did not affect the oxygen absorption; however, it increased reduction processes. Interpolyelectrolyte complex V-asODNs increased the cell respiration by 1.95 times. VI separately increased the cell absorption of oxygen by 18 % and in the complex with asODNs — by 36 %. VI possessed the lowest cytotoxicity. **Conclusions.** New cationic polyelectrolytes form complexes with asODNs. VI causes the smallest effect on the RedOx processes of model cells and possesses the lowest cytotoxic effect.

**Keywords:** antisense oligodeoxynucleotides, polyelectrolyte complexes, cytotoxicity, redox processes, prion.

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Introduction

The pathogenesis of prion infections is associated with the synthesis and aggregation of the cellular form of prion (PrP<sub>C</sub>). Therefore, it is assumed that the elimination or reduction of PrP<sub>C</sub> synthesis in the body will prevent the development and manifestation of transmissible spongiform encephalopathy [1–2].

The possible ways to inhibit the protein synthesis include the use of antisense oligodeoxynucleotides (asODNs), which are complementary to a specific region of mRNA [2–4]. Antisense technology allows “turning off” the gene temporarily, which is useful for the development of an adequate strategy for the prevention and treatment of prion infections. Noteworthy, the nucleic acids due to the nucleases degradation activity and poor cellular delivery does not penetrate the cell membrane, which imposes serious restrictions on their use. The success of the use of antisense technology largely depends on the availability of appropriate carriers for asODNs. Cationic synthetic and natural polymers can be carriers for the delivery of genetic material to cells because through electrostatic interactions they effectively bind negatively charged DNA molecules [5]. Thus, they shield DNA from the enzyme degradation, prolong the residence time of genetic constructs in the body and facilitate their penetration into the cells [4]. This invented scheme for inhibition of the development and prevention of prion infections also requires constant drug administration [2, 4, 6]. Prolonged administration of the active substances likely alters the processes associated with ATP resynthesis and protein phosphorylation in cells. The alteration of redox homeostasis is recognized as a significant cause of male factor infertility. Many factors can deregulate this complex network in animals, including exposure to chemicals, toxins and diseases [7].

The aim of the study was to evaluate qualitatively the ability of polymeric carriers to bind antisense oligodeoxynucleotides, and to investigate their effect on the cell oxidative processes in vitro.

Materials and Methods

Solvents: dimethylformamide (DMF), 1,4-dioxane, acetone, n-hexane were purchased from Merck (Darmstadt, Germany). Monomers: 2-(dimethylamino)ethyl methacrylate (DMAEM), 2-aminoethyl methacrylate hydrochloride (AEM), butyl acrylate (BAC), N-vinylpyrrolidone (NVP) were received from Sigma Aldrich and used without purification. Azoisobutyronitrile (AIBN), poly(ethylene glycol), Mn = 550 (PEG), ammonium cerium (IV) nitrate were received from Aldrich Chemical and used without purification. Peroxy-containing chain transfer agent 1-isopropyl-3-(4)-(1-(tert-butylperoxy)-1-methylethyl]benzene (MP) was synthesized as described earlier [8].

NaF, amytal, sodium azide, disodium ethylenediaminetetraacetic were purchased from Merck (Darmstadt, Germany). Synthesis of poly(DMAEM)-MP (I). AIBN (2 mmol) was dissolved in ethyl acetate (15 mL). DMAEM (112 mmol) and MP (30 mmol) were added, and the mixture was purged with Argon. Polymerization was carried out at 70 °C for 6 h. The conversion was 65–70 %, as determined from dilatometer and
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gravimetric measurements [9]. The target product was precipitated from acetone into hexane three times and dried in vacuum.

In $^1$HNMR, the DMAEM units offered the following signals: skeletal $-\text{CH}_2-$ at 1.75 ppm; fragments $-\text{N(C(H}_3)_2$ at 2.30 ppm; $-\text{CH}_2$–$\text{N(CH}_3)_2$ at 2.55–2.62 ppm; $-\text{C(O)}$–$\text{CH}_2-$ at 4.2 ppm and $\text{CH}_3$–$\text{C}$– at 1.32 ppm. Signals from the benzene ring at 7.05–7.22 ppm and the t-butyl group at 1.14–1.18 ppm indicated availability of terminal peroxide fragment.

Synthesis of polyDMAEM-co-BAC)-MP (II). AIBN (2 mmol) was dissolved in ethyl acetate (15 mL). DMAEM (112 mmol), BAC (10 mmol) and MP (30 mmol) were added, and the mixture was purged with Argon. Polymerization was carried out at 70 °C for 6 h. The conversion was 65–70 %. The target product was precipitated from acetone into hexane three times and dried in vacuum.

In $^1$HNMR, the DMAEM units offered the following signals: skeletal $-\text{CH}_2-$ at 1.75 ppm; fragments $-\text{N(C(H}_3)_2$ at 2.30 ppm; $-\text{CH}_2$–$\text{N(CH}_3)_2$ at 2.55–2.62 ppm; $-\text{C(O)}$–$\text{CH}_2-$ at 4.2 ppm and $\text{CH}_3$–$\text{C}$– at 1.32 ppm. The BAC units signal is $-\text{O}$–$\text{C}$– at 3.95 ppm. The presence of the terminal peroxide group is confirmed by signals of protons of the benzene ring (7.22 and 7.14 ppm), t-butyl $\text{C(CH}_3)_2$OOC($\text{CH}_3)_3$ (1.1 ppm) and $\text{C(CH}_3)_2$OOC($\text{CH}_3)_3$ (1.50 ppm).

Synthesis of polyDMAEM)-block-poly(NVP-co-BAC-co-AEM) (III). Synthesis of the block copolymers was carried out via solution polymerization of the NVP, BAC, and AEM initiated by I. Monomer mixture NVP (110 mmol), BAC (27 mmol) and AEM (11 mmol) were added to the solution of pDMAEM-MP (0.45 g) in DMF (15 mL). Then the mixture was stirred at 80 °C for 24 h under the Argon atmosphere. The resulting block copolymers were precipitated three times from acetone to hexane, separated, purified via dialysis and dried under vacuum.

$^1$H NMR of the III: the polymeric skeletal $-\text{CH}_2-$ at 1.92–1.75 ppm; the DMAEM fragments units demonstrated the following signals: $-\text{N(C(H}_3)_2$ at 2.30 ppm, $-\text{C(O)}$–$\text{CH}_2-$ at 4.1–4.1 ppm, $\text{CH}_3$–$\text{C}$– at 1.34 ppm and $\text{Ar}$–$\text{H}$ at 7.05–7.20 ppm; the poly(NVP-co-BAC-co-AEM) fragments units demonstrated the following signals the VP units: skeletal–$\text{CH}_2-$ at 1.23 ppm, skeletal–$\text{CH}_2-$ at 3.64 and 3.79 ppm; pyrrolidone ring: a–$\text{CH}_2-$ 3.37 ppm, b–$\text{CH}_2-$ 2.1 ppm, c–$\text{CH}_2-$ at 2.25 and 2.35 ppm; signals the BAC units:–$\text{O}$–$\text{C}$– 3.95 ppm; signals the AEM units: $\text{NH}_2$–$\text{CH}_2$–$\text{CH}_2$–$\text{O}$ at 3.03 ppm and $\text{NH}_2$–$\text{CH}_2$–$\text{CH}_2$–$\text{O}$ at 4.1 ppm.

MP-poly(DMAEM)-PEG-poly(DMAEM)-MP (IV-VI). Tri-block-copolymers IV-VI were synthesized via polymerization in water in the flask protected from light at 291 K. DMAEM (0.45 mol/L), 0.08 mol/L PEG, MP and concentrated nitric acid were dissolved in 20 mL of solvent. The mixture was purged by argon. The reaction was started by injecting the Ce(IV) salt (0.076 mol/L) dissolved in 5 mL solvent. Polymerization was carried out for 4 h. The polymer was washed with acetone and dried under vacuum. Polymer V was not purified from Ce ions.

$^1$HNMR of polymers: PEG fragments are confirmed by signals at 3.51 and 3.44 ppm corresponding to protons of $\text{CH}_2$-groups of the main chain. DMAEM link: the protons of the methyl groups — $\text{CH}_2$–$\text{N(C(H}_3)_2$ appear as an intense peak at 2.79 ppm; a peak at 4.23 ppm corresponds to methylene groups — $\text{CH}_2$–$\text{CH}_2$–$\text{N(C(H}_3)_2$; the signals of the protons of the...
side methyl group — CH₂-C(CH₃) — appear in the region of 1.18 ppm, and the protons of the methylene group of the main chain — at 1.91 ppm. The presence of the peroxide group is confirmed by signals of the benzene ring (7.22 and 7.14 ppm), tert-butyl C(CH₃)₂OOC(CH₃)₃ (1.1 ppm) and C(CH₃)₂OOC(CH₃)₃ (1.50 ppm).

The asODNs-polymer complexes were synthesized by the method [10]. Polymer (8 mg) was dissolved in 0.01M HCl, pH of the solution 7.4 and volume 10 ml. 0.5 mL of polymer solution were mixed with 0.5 mL of aqua solution of asODNs (C = 20 nmol/mL). Mixtures were incubated for 30 min (298 K).

Polymer cytotoxicity. The effect of polymers and their complexes with asODNs on the cell survival and oxidative processes was studied in vitro on sperm. The ejaculates were collected from bulls: V = 2–4 mL; C = 0.8–1.3·10⁹ cells/mL; vitality = 70 % or more. To the sperm suspension (200 μL) 10 μL of polymers in concentrations of 0.05 %, 0.5 % and 1 % were added. Sperm survival (h) was assessed under a microscope (×200) until the cessation of rectilinear translational motion [5].
Detection of complexes formation as ODNs-polymers. The ability of polymers to bind oligonucleotides was determined by the method of free diffusion in agarose gel [5, 11].

Cellular metabolic activity was determined for the cells after 96 hours of the cultivation. The intensity of oxygen consumption by cells was established polarographically (ng-atoms O$_2$·min$^{-1}$·mL$^{-1}$·cell suspension) using the Clark electrode, which was mounted in a thermostated container (t = 38.5 °C). The cell respiration was measured using standard polarographic techniques with a Clark-type electrode. Cell reduction processes were measured potentiometrically (mV/min/10$^6$ cells) [12] using open system of microelectrodes [13]. The fraction of oxygen consumed by aerobic glycolysis was determined using an inhibitor of the specified metabolic pathway (NaF; 10$^{-3}$ M). The fraction of oxygen consumed by the NAD-dependent chain of the electron transport chain was detected using amytal (5·10$^{-3}$ M) and sodium azide NaN$_3$ (5·10$^{-2}$ M). The intensity of free radical oxidation of unsaturated fatty acids was determined using Na$_2$EDTA (0.6·10$^{-3}$ M). The study of the specific oxygen uptake rate of cells was carried out at 38.5 °C in phosphate-buffered saline (PBS: NaCl — 0.8 g; KCl — 0.02 g; Na$_2$HPO$_4$ — 0.11 g; KH$_2$PO$_4$ — 0.02 g; MgCl$_2$ — 0.01 g; H$_2$O to 100 mL; pH — 7.4). 0.9 mL of PBS and 0.1 mL of cell suspension were introduced into the thermostatic container.

Result and Discussion

We measured the redox processes caused by the polymers and their complexes with therapeutic asODNs, which decrease the PrP$^C$ level [5]. The prion-infected transgenic mice overexpressing the hamster prion protein (Tg7 mice) suffer from mitochondrial respiratory deficits [14]. Mitochondrial respiration of cells/tissues is a significant proportion of total oxygen uptake. The cell respiration involves oxidation and reduction processes (redox). It was found that the polymers increase cel-
In order to establish the effect of active substances on cell metabolism and ATP resynthesis, the intensity of substrate use and electron transport in the mitochondrial respiratory chain was assessed. In particular, NaF inhibits the conversion of phosphoglyceric acid into phosphopyruvic acid and therefore arrests glycolysis [15], amital inhibits the I complex of mitochondrial electron transport chain [16, 17], NaN₃ is an inhibitor of cytochrome oxidase [18, 19]. The inhibitors cause a gradual decrease of oxygen uptake. At the same time, depending on the intensity of electron transport in the respiratory chain, there are different magnitudes of the flow of electrons/protons into the extracellular space and, therefore, different reduction processes. During addition of inhibitors, the polymers depress both the use of substrates and the transport of electrons in the mitochondrial respiratory chain to a greater extent than in control. In particular, NaF together with polymer I reduce respiratory activity by 2.2 times (p <0.05), and by 40.4 % (p <0.001) in control (without polymers). Sequential addition of inhibitors: amytal, NaN₃ and Na₂EDTA gradually reduces the oxygen consumption by cells, respectively, by 44.5 %, 33.3 % and 43.0 %. Similarly, the reduction ability of cells decreases: by 32.0 % under NaF influence, by 19.2 % — under amytal and by 51.0 % — under NaN₃, but adding Na₂EDTA causes an increase by 14.8 % (Fig. 3). The effect of NaF under the action of II is manifested by 42.4 % decrease of the oxidation process, whereas the sequential addition of amytal, NaN₃ and Na₂EDTA inhibits this process by 12.7 times. The reduction processes of cells with the ad-

Fig. 3. Redox processes in cells under the action of polymer I, II and III.
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dition of II and the use of inhibitors of both glycolysis and mitochondrial respiratory chain were almost unchanged. The range was from 3.7 to 5.3 mV/min/10^6 cells. Na_2EDTA decreased oxygen uptake by 49.1 % (p <0.05) compared to the initial value (Fig. 3). Polymer III reduced redox potentials (Fig. 3). The analysis of the effect of polymers on the respiratory activity of cells in vitro shows that the polymer I causes the accumulation of protons in the extracellular space, but the penetration into the cells has low impact on the oxidation processes. Polymer II apparently penetrates the membranes and activates oxygen consumption and almost does not change the outflow of protons from cells. Polymer III stimulates both oxidation processes and the flow of electrons into the extracellular space. Polymer I inhibits the aerobic glycolysis and increases the sensitivity of this process to the influence of the inhibitor. Inhibition of aerobic glycolysis is accompanied by a double accumulation of protons in the extracellular space. By adding polymer II, the effect of inhibitors on the ATP resynthesis is similar to the control. However, during the inhibition of the NAD-dependent link in the mitochondrial respiratory chain, the flow of electrons increases in contrast to the control, where an increase of the space potential was found. Polymer III, after activation of oxidation processes, reduces the sensitivity to the inhibitors of both aerobic glycolysis and electron transport links in the mitochondrial respiratory chain.

Polymers IV and VI, which are derived from polymer I and contain PEG, reduce the cell oxygen uptake by 53.2 % and 34.6 %, respectively. Polymer V increases the cell oxygen uptake by 23.9 % (Fig. 4 A).
The presence of cerium ions in the V affects the membrane structure of cells and modifies them [20, 21], which provides penetration and use of exogenous substrates. The addition of IV to the sperm suspension stimulated (or caused) the flow of positively charged particles (protons) into the extracellular environment and, conversely, the additions of VI and V were characterized by the changes in cell metabolism, accompanied by the formation of negatively charged particles and their accumulation in the extracellular space.

IV–asODNs and VI–asODNs complexes do not change the intensity of cellular respiration, compared with separately added polymers. We suggest that the reducing of the im-
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Impact on cells by these polymers in combination with asODNs happens due to already bound active groups of the polymers. The cell membranes of mammalian are negatively charged [22]. The free active groups of cationic polymers obviously are able to interact with the components of negatively charged cell membranes, change their structure, disrupt integrity and, accordingly, have a stronger effect on redox processes in cells. However, the V–asODNs complex, apparently due to the modification of membranes, penetrates into the cells and reduces the redox processes. With the addition of IV the potential of cell medium increased by 2.5 times, and with V and VI decreased equally by 30.3 %, compared to the control. IV–asODNs complex does not change the cell oxidation, compared to IV without asODNs, but it increases the reduction processes (fig. 4 A). Similarly, the cell oxidation processes were at the same level during addition of VI–asODNs complex or polymer VI (0.5 ng-atom O2/min/106 cell), and the reduction processes increased to 3.0 mV/min/106 cell (Fig. 4 C). V–asODNs complex reduces both cell oxygen uptakes by 45.2 % and cell reduction processes by 96.7 %, compared to separately added V (Fig. 4 B).

After the addition of NaF to cultural mediums with IV and IV–asODNs, the absorption of oxygen decreased by 2.1 and 8.2 times respectively. The addition of NaF also reduces the respiratory activity together with the influence of other polymeric compounds and their complexes with asODNs. During the addition of amital to the cell medium with IV or with IV–asODNs complex, the formation of oxygen in a polarographic container was detected. At the same time, in the presence of IV the flow of charged particles is almost equal to 0 (0.001 mV/min/106 cell). Complexes IV–asODNs and VI–asODNs stimulate an increase of space potential, respectively, by 29.4 and 80.0 %. During the test of VI, the reduction processes do not change (2.0 mV/min/106 cells). Under the action of V and its complex with asODNs the reduction processes decrease by 33.4 and 99.9 %, respectively.

Inhibition of the terminal link of the mitochondrial respiratory chain by NaN3 in the presence of IV–asODNs and V led to the release of oxygen into the cell environment. At the same time, the addition of amytal to the cell with IV and VI almost completely inhibits the flow (outflow) of charged particles (protons and electrons) from the NAD-dependent path of electron transport in the mitochondrial electron transport chain, which is manifested by a decrease of the potential to zero. The addition of amytal to the cell with V does not change the potential (2.0 mV/min/106 cells), and in the presence of both IV–asODNs and VI–asODNs decreases it. The addition of Na2EDTA to cells with polymers both alone and in complexes with asODNs leads to an inhibition of reduction processes. It was detected that IV and IV–asODNs mostly inhibit aerobic glycolysis and stimulate the flow of protons into the extracellular space. These compounds together with amytal stimulate the generation of oxygen in a polarographic container. It is known that the blocking of the electron transport can lead to the formation of O2•− [23–25]. Probably, the polymer IV and the complex IV–asODNs change the structure of membranes, penetrate into cells and enhance the inhibitory action of amytal. Generation of the active form of oxygen (O2•−) by the mito-
Chondrial respiratory chain is one of the causes of membrane destructions [26]. Therefore, the use of IV and V, both alone and in combination with asODNs, can cause changes in cell structure and stimulate the processes of free radical oxidation and $O_2•−$ formation. However, the effect of these compounds on the structures and metabolism is “mild”, as their action ensures the survival of cells for 96 hours. The least toxic for cells is the polymer VI and its complex with asODNs. These compounds support aerobic glycolysis, with a weak effect on individual links of electron transport in the mitochondrial respiratory chain. Obviously, after penetration through the membrane into the cell, the polymer VI and VI–asODNs complex are able to normalize the use of endogenous substrates and provide resynthesis of ATP, high maintaining mobility and survival for 120 hours.

Conclusions
Polycationic polymers, which are based on DMAEM, form the complexes with prion inhibitors — antisense oligodeoxynucleotides. Polymers differed in their effect on REDOX processes and cytotoxicity. Polymer VI is characterized by the lowest cytotoxicity and minimal effect on redox processes in cells. Polymer VI is the most suitable transporter of asODNs for inhibition of the synthesis of physiological prion in animal cells.

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Вплив комплексів терапевтичних антисенс-олігодезоксинуклеотидів з катіонними полімерами на дихання клітин

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Антисенс-ДНК технології — це нова стратегія лікування пріонних інфекцій. Ця стратегія вимагає тривалого введення ліків, які, ймовірно, змінюють окисно-відновні процеси в клітинах. Мета. Оцінка виживання клітин та інтенсивності окисних процесів in vitro під впливом антисенс-олігодезоксинуклеотидами (асОДН) як інгібіторів фізіологічного пріону (PrPC) у комплексі з катіонним поліелектролітом. Методи. Дослідження утворення комплексів між полімерами різної природи та асОДН — шляхом вільної дифузії в гелі агарози, цитотоксичною активністю — на модельних клітинах (спермії бугая), поглинання кисню й відновну здатність клітин — полярографічно та потенціометрично, аналіз результатів дослідження — статистично. Результати. Встановлено, що полікатіонні полімери на основі диметиламіноетилметакрилату (DMAEM) утворюють комплекси з асОДН. Було встановлено, що всі досліджувані полімери, які містять поліетиленгліколь (ПЕГ), збільшують поглинання кисню клітинами: на 18 % (VI), на 37 % (IV) і в 2,6 рази для V. Додавання в клітинне середовище комплексу IV–асОДН не впливало на поглинання кисню, а посилювало процеси відновлення. Полімерний комплекс V–асОДН збільшив дихання клітин в 1,95 рази, що нижче, ніж окрема дія V на ці клітини. Полімер VI окремо збільшував клітинну абсорбцію кисню на 18 %, а в комплексі з асОДН — на 36 %. Полімер VI продемонстрував найнижчу цитотоксичність. Висновки. Розроблено нові катіонактивні полімери, які утворюють комплекси з асОДН. Найменшій вплив на окисно-відновні процеси моделюних клітин (спермії) чинить сполука полімерної природи VI, для якої характерна низька цитотоксична дія in vitro.
Вплив комплексів терапевтичних антисенс-олігодезоксинуклеотидів з катіонними полімерами на дихання клеток

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Ан-тисенс-ДНК технології — це нова стратегія лікування протеїнопатій, таких як прионні інфекції, болізнь Альцгеймера і інші. Ця стратегія потребує тривалого введення ліків, які, зважаючи на, змушують кислотно-відновні процеси в клітинах.

Ціль. Оцінка живкості клітін і інтенсивності окислительно-восстановлювальних процесів in vitro під впливом антисенс-олігодезоксинуклеотидів (асОДН) як інгібіторів фізіологічного приона (PrPС) в комплексі з катіонними поліелектролітами. Методи. Ізучення цитотоксичністі на моделюючих клітинах (сперматозоїди кравця), оцінка здатності комплексів між полімером і асОДН через свободну дифузію в гелі агарози, полярографічне і потенціометричне зорієнтування поглощення кисню клітінами, статистичний аналіз результатів дослідження. Результати. Установлено, що полікатаонні полімери на основі диметиламіноетилметакрилату (ДМААМ) утворюють комплекси з асОДН. Установлено, що все вивчені полімери, які містять поліетиленгліколь (ПЕГ), збільшують поглинання кисню клітінами: на 18% (VI), на 37% (IV) і в 2,6 раза (V). Додавання в клітинну рідину комплекса IV–асОДН не вплинуло на поглинання кисню клітінами, але збільшило окисні процеси. Полімерний комплекс V–асОДН збільшив дихання клітін в 1,95 раза, що нижче, ніж здійснення окиснительно-восстановлювальних процесів моделюючих клітін.

Висновки. Розроблені нові катіонні полімери, здатні формувати комплекси з асОДН. Полімер VI відображає на мінімальній інтенсивності окиснительно-восстановлювальних процесів моделюючих клітін.

Ключові слова: антисенс-олігодезоксинуклеотиди, комплекси поліелектролітів, цитотоксичність, окиснительно-восстановлювальний процес, прион.

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