УДК 615.277.3.015.14:546.72-022.532
https://doi.org: 10.20538/1682-0363-2019-2-69–79

Development and properties of a new doxorubicin carrier based on surface-modified iron zero-valent microparticles with high encapsulation efficiency and the possibility of its controlled release

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ABSTRACT

Currently, chemotherapy combined with surgery and radiation therapy is the most effective treatment for cancer. At the same time, the use of this method is accompanied by serious side effects caused by the lack of specificity of most chemotherapeutic agents. In this regard, the development of drug delivery systems (DDS) capable of addressing a chemotherapeutic agent to cancer cells, as well as its controlled release, is a promising approach for the effective treatment of cancer.

The aim of the study is to synthesize a new DDS based on surface-modified microparticles of zero-valent iron, to study its properties as a carrier of a chemotherapeutic agent (encapsulation efficiency, loading capacity, possibility of controlled release of a chemotherapeutic agent) and safety.

Materials and methods. The microparticles were synthesised by reduction of iron (III) chloride with sodium borohydride followed by *in situ* surface modification by 4-carboxybenzyldiazonium tosylate. To confirm the occurrence of the reaction, FTIR spectroscopy (Nicolet iS5 Infrared Spectrometer (Thermo Scientific, USA)) was used. Hydrodynamic diameter and surface charge of the microparticles in solution were investigated by dynamic light scattering (DLS) and z-potential. DOX release studies were performed in simulated physiological conditions (pH 3.3; 5.5; 7.4) to evaluate the effect of the external pH on the release rate. Release studies under ultrasound irradiation were performed simultaneously in the same conditions. The effect of surface modification on encapsulation efficiency was evaluated at various pH values (3.3; 5.5; 7.4) and doxorubicin concentrations (0.2; 0.35; 0.5; 0.75; 1.0 mg/ml). To demonstrate the safety of the developed system, cytotoxicity studies were performed on HeLa cell lines (ATCC® CCL¬2™).

Results. An original method of preparation of the drug carrier, based on iron zero-valent microparticles with covalently attached chitosan (Fe-CS) on their surface was proposed. Prepared

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microparticles demonstrated high encapsulation efficiency, drug loading capacity of DOX (0.9 mg per 1 mg of Fe-CS microparticles), low cytotoxicity and also a possibility to modulate the release rate by ultrasound irradiation and by changing pH of the external environment.

Conclusion. A carrier based on microparticles of zero-valent iron with covalently attached to the surface chitosan (Fe-CS) was obtained. The efficiency of encapsulation, the loading capacity of doxorubicin was determined and the possibility of its controlled release under the influence of an ultrasonic field at different pH values was confirmed. In an *in vitro* experiment on the HeLa cell line (ATCC® CCL¬2™), no toxicity was established for all samples (Fe^0, Fe-COOH и Fe-CS), regardless of their concentration.

Key words: doxorubicin, chitosan, zerovalent iron microparticles, drug delivery, stimuli responsive carrier, controlled release.

Conflict of interest. The authors declare the absence of obvious and potential conflicts of interest related to the publication of this article.

Source of financing. The study was supported by the TPU development project.

For citation: Di Martino A., Vlasov S.S., Guryev A.M., Yusubov M.S., Postnikov P.S., Belousov M.V. Development and properties of a new doxorubicin carrier based on surface-modified iron zero-valent microparticles with high encapsulation efficiency and the possibility of its controlled release. *Bulletin of Siberian Medicine*. 2019; 18 (2): 69–79. https://doi.org: 10.20538/1682-0363-2019-2-69–79.

УДК 615.277.3.015.14:546.72-022.532
https://doi.org: 10.20538/1682-0363-2019-2-69–79

Разработка и свойства нового носителя доксорубицина на основе поверхностно-модифицированных микрочастиц ноль-валентного железа с высокой эффективностью инкапсуляции и возможностью его контролируемого высвобождения

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РЕЗЮМЕ

В настоящее время химиотерапия в сочетании с хирургией и лучевой терапией является наиболее эффективным методом лечения рака. В то же время применение данного метода сопровождается серьезными побочными эффектами, обусловленными неспецифичностью большинства химиотерапевтических агентов. В связи с этим разработка систем доставки лекарственных средств (СДЛС), способных обеспечить адресацию химиотерапевтического

агента к раковым клеткам, а также его контролируемое высвобождение представляют собой перспективный подход для эффективного лечения онкологических заболеваний.

Цель работы – синтез нового СДЛС на основе поверхностно-модифицированных микрочастиц ноль-валентного железа, изучение его свойств в качестве носителя химиотерапевтического агента (эффективность инкапсуляции, емкость загрузки, возможность контролируемого высвобождения химиотерапевтического агента) и безопасности.

Материалы и методы. Частицы были получены методом восстановления хлорида железа (III) боргидридом натрия с последующей *in situ* модификацией поверхности 4-карбоксибензолдиазония тозилатом согласно модифицированной методике. Наличие функциональных групп на поверхности подтверждали методом ИК-спектроскопии с использованием спектрометра Nicolet iS5 Infrared Spectrometer (Thermo Scientific, США). Размеры и поверхностный заряд микрочастиц в растворе исследовали методом динамического рассеяния света и дзета-потенциала. Для оценки влияния pH окружающей среды на скорость высвобождения доксорубицина исследование проводили в моделированных физиологических условиях (pH 3,3; 5,5; 7,4). Изучение высвобождения под воздействием ультразвукового поля проводили одновременно при тех же условиях. Влияние модификации поверхности на эффективность инкапсуляции оценивали при различных значениях pH (3,3; 5,5; 7,4) и концентрациях доксорубицина (0,2; 0,35; 0,5; 0,75; 1,0 мг/мл). Для подтверждения безопасности разработанной СДЛС определение цитотоксичности проводили на клеточной линии HeLa (ATCC® CCL-2™).

Результаты. Предложена оригинальная методика получения носителя на основе микрочастиц ноль-валентного железа с ковалентно присоединенным к поверхности хитозаном (Fe-CS), обладающего высокими значениями эффективности инкапсуляции и емкости загрузки доксорубицина (0,9 мг на 1 мг микрочастиц Fe-CS), низкой цитотоксичностью, а также возможностью контролируемого высвобождения цитостатического агента (доксорубицина) под воздействием ультразвукового излучения при различных значениях pH.

Заключение. Получен носитель на основе микрочастиц ноль-валентного железа с ковалентно присоединенным к поверхности хитозаном (Fe-CS). Определена эффективность инкапсуляции, емкость загрузки доксорубицина и подтверждена возможность его контролируемого высвобождения под воздействием ультразвукового поля при различных значениях pH. В эксперименте *in vitro* на клеточной линии HeLa (ATCC® CCL-2™) установлено отсутствие токсичности для всех образцов (Fe^0, Fe-COOH и Fe-CS) вне зависимости от их концентрации.

Ключевые слова: доксорубицин, хитозан, микрочастицы ноль-валентного железа, доставка лекарственных средств, стимул-чувствительный носитель, контролируемое высвобождение.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Источник финансирования. Работа выполнена при финансовой поддержке проекта развития ТПУ.

Для цитирования: Ди Мартино А., Власов С.С., Гурьев А.М., Юсубов М.С., Постников П.С., Белоусов М.В. Разработка и свойства нового носителя доксорубицина на основе поверхностно-модифицированных микрочастиц ноль-валентного железа с высокой эффективностью инкапсуляции и возможностью его контролируемого высвобождения. *Бюллетень сибирской медицины.* 2019; 18 (2): 69–79. https://doi.org: 10.20538/1682-0363-2019-2-69–79.

INTRODUCTION

Despite the fact that chemotherapy is one of the primary [1] and the most effective methods of treatment of malignant tumors, its use is still limited to serious side effects [2, 3]. One of the causes of side effects is the lack of specificity of chemotherapeutic agents to the tumor, with the result that their administration leads to toxic effects on healthy cells of the body [4]. Thus, in addition to reducing the quality of life of patients, the side effects of chemotherapy remain a very serious obstacle to its successful clinical use [5].

One of the ways to resolve this problem is the use of drug delivery systems (DDS). Thanks to these systems, it became possible to significantly reduce the number of side effects of chemotherapy, as well as the use of new, more effective treatment regimens [6], since DDSs provide easy administration of the drug, as well as an increase in its accumulation in the tumor [7-9]. At the same time, the main disadvantages of most DDSs may be the low content of the active substance, the impossibility of remote initiation of its release, as well as the low encapsulation efficiency of the therapeutic agent.

There are a number of studies dedicated to overcoming these drawbacks. For example, the encapsulation of the drug can be increased by using amphiphilic polypeptides [10] or oligonucleotides [11], but using chitosan as a modifier of the carrier surface may allow an increase in the loading of the chemotherapeutic agent due to the possibility of formation of a large number of hydrogen bonds, as well as physical interactions [12] due to the uniqueness of its polymeric structure. Doxorubicin is planned to be used as a model chemotherapeutic agent, the binding of which to the surface of the carrier occurs due to electrostatic interactions. Since positively charged amino groups are present in doxorubicin and chitosan molecules [13–16], it is advisable to use a negatively charged crosslinker, such as sodium tripolyphosphate [17], for electrostatic binding of these system components. The advantages of such a DDS are the higher encapsulation efficiency and, consequently, an increase in the content of the therapeutic agent, as well as the ability to control the kinetics of its release due to exposure to both external (ultrasonic radiation) and internal factors (environmental pH values). Moreover, zero-valent iron particles are a promising carrier for the development of effective DDS due to their better magnetic properties as compared to iron oxides [18], and the use of micron-sized particles allows solving local tumor embolization problems for therapeutic purposes.

Thus, the purpose of this study is the synthesis of a new DDS based on surface-modified microparticles of zero-valent iron, the study of its properties as a carrier of a chemotherapeutic agent (encapsulation efficiency, loading capacity, possibility of controlled release of a chemotherapeutic agent) and safety.

MATERIALS AND METHODS

For this research, reagents and organic solvents were used, which are marketable products of Aldrich (USA), Fluka and others, of appropriate purity, and were used without prior purification.

Determination of the size and zeta potential of microparticles was carried out using an aqueous suspension with a concentration of 1 mg / ml and pH = 7 on a Zetasizer Nano ZS instrument (Malvern, United Kingdom). To determine the size, dynamic light scattering was used. The evidence of covalent grafting of organic functional groups on iron microparticles (Fe-COOH) was evaluated by IR spectroscopy using a Nicolet iS5 Infrared Spectrometer (Thermo Scientific, United States).

The method of the synthesis of 4-carboxybenzenediazonium tosylate. Synthesis was carried out in accordance with the method [19].

The method of synthesis of microparticles Fe-COOH and Fe-CS. The synthesis of iron microparticles was carried out according to a modified synthesis method developed earlier [20]. Iron (III) chloride (0.406 g; 1.5 mmol) and sodium borohydride (0.171 g; 4.5 mmol) were dissolved in 10 ml of distilled water. Next, in a three-necked flask in an argon atmosphere, 5 ml of the prepared solutions were mixed. Stirring was carried out for 10 minutes using a magnetic stirrer. Then, the remaining 5 ml of iron (III) chloride and sodium borohydride solutions were added to the resulting mixture and mixed again for 10 minutes. Next, 20 ml of an aqueous solution of 4-carboxybenzenediazonium tosylate (0.3 g) was added to the resulting reaction mass (0.3 g) and stirring continued for another 40 minutes. The resulting Fe – COOH microparticles were separated from the mixture by precipitation with a neodymium magnet and successively washed with water, ethanol, and acetone until a clear solution was obtained over the particles. After washing, the microparticles were freeze-dried to remove traces of solvents.

To obtain Fe-CS microparticles, a portion of the Fe-COOH microparticles, weighing 75 mg, obtained in the previous step, were suspended in 75 ml of water. 12.9 mg of N- (3-dimethylaminopropyl) -N'-ethylcarbodiimide hydrochloride and 15.54 mg of N-hydroxysuccinimide were added to the suspension. The mixture was stirred for 2 hours. In parallel, 750 mg of chitosan was dissolved in 300 ml of 1% (v / v) acetic acid. Next, the Fe-COOH suspension was added to the chitosan solution. The resulting mixture was left under thorough stirring for 48 hours.

Then, to isolate Fe-CS microparticles, the resulting mixture was centrifuged at 7500 rpm for 5 min. The supernatant was separated, and the resulting precipitate was resuspended in 50 ml of distilled water and centrifuged under the same conditions. The washing procedure was repeated 3 times. Purified Fe-CS microparticles were freeze-dried.

The method of obtaining conjugate Fe-CS / dox. A portion of 20-mg Fe-CS microparticles was suspended in 20 ml of water. In parallel, 20 ml of doxorubicin (DOX) and sodium tripolyphosphate (STP) solutions with concentrations of 1 mg / ml were prepared. Both solutions were mixed and left under stirring for 1 h on a magnetic stirrer.

Next, the resulting mixture DOX/STP was added dropwise to a suspension of Fe-CS microparticles. A mixture of Fe-CS/STP/DOX was left under thorough stirring for 2 hours.

The resulting reaction mass was centrifuged at 7500 rpm for 5 minutes. The supernatant was separated, and the residual matter of microparticles was re-suspended in distilled water and centrifuged under the same conditions, and then freeze-dried.

Study of doxorubicin release and encapsulation effectiveness. The release study was carried out at a constant temperature of 37°C and stirring at 100 rpm. For the experiment the incubator Stuart SI 500 (Stuart, UK) was used. The solvent used was a mixture of KCl/HCl with an initial pH value of 3.3. The mixture was prepared by mixing 50 ml of a 0.2 M KCl solution with 140 ml of distilled water. Next, 0.2 M hydrochloric acid solution was added until the pH was 3.3. Then the resulting solution was adjusted to 400 ml with distilled water. The release studies were performed at three different pH values (3.3; 5.5; 7.4). The value of acidity values were changed sequentially. For the experiment, 10 ml of a 1 mg/ml Fe-CS/DOX conjugate suspension was prepared. The test sample was placed in an incubator and, at set intervals, samples of the release medium were taken in a volume of 2 ml. The sample was pre-centrifuged at 7,500 rpm for 5 minutes. The concentration of released doxorubicin in the sample was determined by UV spectroscopy at 480 nm, using an Evolution 201/220 UV-Visible Spectrophotometers (Thermo Scientific, United States). The selected volume of medium was replaced with an equivalent volume of fresh KCl/HCl solution. The change in pH values was performed by adding 0.1 M sodium hydroxide solution to achieve the required value.

The study of the release of doxorubicin under the influence of ultrasound was performed in parallel under conditions similar to those described above. The difference of this technique is the introduction of the test sample into an ultrasonic field with a frequency of 75 kHz and a specific power density of 2 W/cm^2 for 30s. An ultrasonic bath Elmasonic S10H (Elma, Germany) was used as a source of ultrasonic radiation. The sonication was performed immediately prior to centrifuging the sample. The concentration of released doxorubicin in the sample was also determined by UV spectroscopy at 480 nm.

The use of low-frequency ultrasound radiation is necessary to initiate the release of doxorubicin without damaging the structure of body tissues. The described value of the ultrasonic radiation frequency was used to demonstrate the sensitivity of the system under study to external triggering influences.

The efficiency of encapsulation (EI) was determined in accordance with the method [21]. The calculation of EI was performed according to the formula

$$ЭИ\ (\%) = \left(\frac{D_T - D_Ф}{D_T}\right) \times 100,$$

where D_T is the theoretical concentration taken for loading doxorubicin, $D_Ф$ is the concentration of DOX after the encapsulation process.

The concentration of doxorubicin in the samples was determined by UV spectroscopy at a wavelength of 480 nm. To calculate the concentration, a calibration curve was used, which was constructed from the results of measuring the absorption of incident light by doxorubicin solutions in the concentration range 3.75–60 μg / ml [22].

The effect of pH and surface modification on the change in EI was evaluated by the following method. A solution of DOX (1 mg/ml, 1 ml) was added to a suspension of zero-valent iron or Fe-

COOH (1 mg/ml, 1 ml). The resulting mixture was stirred for 5 minutes, and then centrifuged at 14,000 rpm for 15 min. The resulting supernatant was collected for analysis. For Fe-CS microparticles, the determination was carried out in accordance with the described method, but using a mixture of DOX/STP, instead of a DOX solution. A mixture of DOX/STP was prepared using doxorubicin solutions with concentrations (0.6; 1.05; 1.5; 2.25; 3.0 mg/ml) in accordance with the section "Method for the preparation of the Fe-CS/DOX conjugate"; this mixture was added to the suspension of microparticles dropwise.

Methodology for the study of cytotoxicity. Determination of cytotoxicity was performed using a HeLa cell line (ATCC® CCL-2 ™). In vitro cell survival was determined by the MTT method in accordance with the following protocol [23]. Cells were cultured in separate media (2.5×10^5 cells/ml) containing zero-valence iron microparticles (Fe^0), Fe-COOH and Fe-CS, respectively, for 24, 48 and 72 hours. Microparticles-free medium was used as a control.

Suspensions of analyzed microparticles with a concentration of 5–100 μg/ml were prepared directly in the medium. Removal of the medium from each cell was performed by the aspiration method. The cells in each cell were washed with 200 μl of phosphate buffer, and then 50 μl of methyl thiazolyl tetrazolium (MTT) solution at a concentration of 1 mg/ml was added to each cell. After 2 hours of incubation, the MTT solution was removed by aspiration, and 50 μl of isopropanol was added to each cell to dissolve the formazan crystals. The optical density was measured three times for each sample at a wavelength of 595 nm using a multi-channel reader (Tecan, Switzerland). Cell survival (%) was calculated as the ratio of average values of optical density for each sample (I_{sample}) and the control sample ($I_{control}$).

$$\text{Cell survival (\%)} = \frac{(I_{sample})}{(I_{control})} \times 100$$

RESULTS AND DISCUSSIONS

At the first stage, we carried out the synthesis of iron microparticles coated with organic functional groups. To this end, we modified the previously published method [20], which involves the reduction of iron from the corresponding trichloride with sodium borohydride in the presence of 4-carboxybenzenediazonium tosylate. To obtain micron-sized particles, we used a two-stage addition of iron trichloride for the growth of particles, and only then added a solution of 4-caboxybenzenediazonium tosylate for modification. The size of the obtained microparticles was controlled using the method of dynamic light scattering (Table 1).

Table

The size and zeta potential of microparticles

Sample name	Particle size, microns	Zeta potential, mV
Fe^0	4,19 ± 0,12	−0,03 ± 0,01
Fe-COOH	4,32 ± 0,18	−18,92 ± 0,81
Fe-CS	4,48 ± 0,28	20,61 ± 1,51

Experiments to determine the size of iron microparticles (Fe^0, Fe-COOH and Fe-CS) showed the absence of significant dispersion of results in the course of the modifications carried out.

The negative value of the zeta potential for Fe-COOH microparticles is explained by the presence of carboxyl groups on their surface. After further modification with chitosan, a sharp shift of the zeta potential occurs in a positive direction, which, on the one hand, is explained by the appearance of positively charged amino groups, and, on the other, by a decrease in the number of free carboxyl groups due to their participation in the formation of an amide bond.

The data obtained indirectly confirm the covalent modification of the surface by 4-carboxyphenyl groups, as well as the secondary inoculation of chitosan on the surface. These facts were also confirmed by IR spectroscopy (Fig. 1).

The spectrum of microparticles Fe-COOH observed absorption peaks at wavelengths n_{OH} 2974, 2900 and $\nu_{C=O}$ 1689 sm^{-1}, indicating the presence of a carboxyl group (-COOH). Absorption at wavelengths ν_{C-C} 1603 and 1585 sm^{-1} confirms the presence of a benzene ring. Thus, the results obtained may indicate the grafting of 4-carboxyphenyl groups on the surface of microparticles [24].

The spectra of Fe-CS microparticles and chitosan were compared with each other. The chitosan spectrum is characterized by the presence of major absorption peaks at wavelengths of 3350 cm^{-1}, 2870 cm^{-1} (ν_{CH}) and 1060 cm^{-1} (ν_{C-O-C}). The

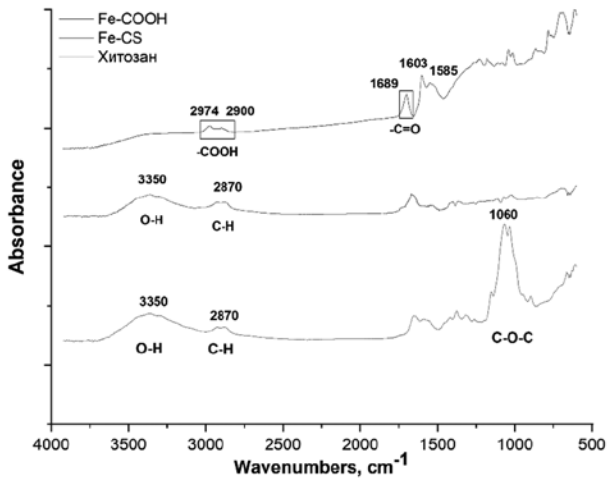

Fig. 1. FTIR spectra of Fe-COOH, Fe-CS microparticles and pure chitosan

Рис. 1. ИК-спектры микрочастиц Fe-COOH, Fe-CS и чистого хитозана

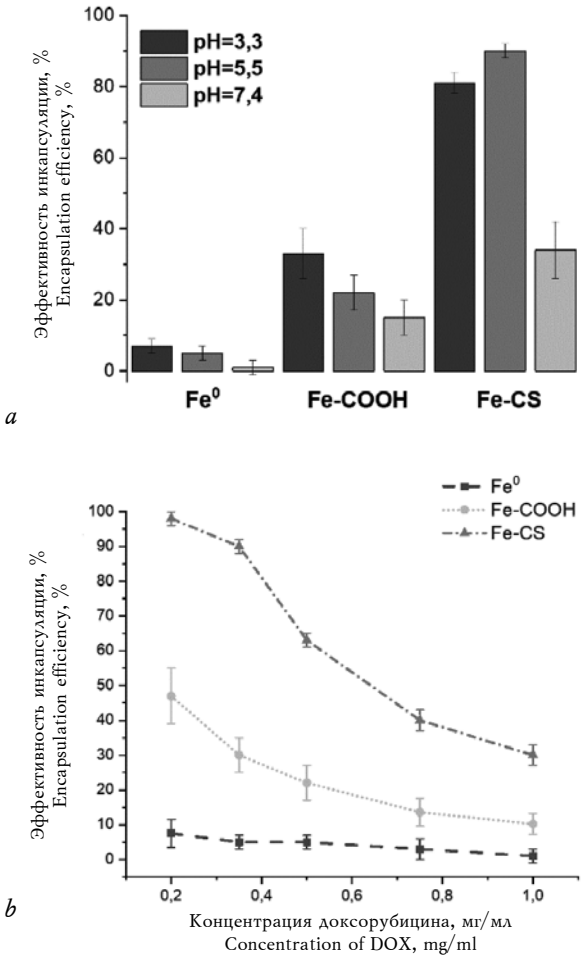

Fig. 2. Influence of pH and surface modification of DOX (*a*) and the concentration of DOX (*b*) on the encapsulation efficiency

Рис. 2. Влияние pH и поверхностной модификации ДОКС (*a*) и концентрации ДОКС (*b*) на эффективность инкапсуляции

spectrum of Fe-CS microparticles also contains absorption bands at wavelengths of 3350 sm^{-1} (n_{OH}) and 2870 sm^{-1} (ν_{CH}). As a result, it can be concluded that chitosan is present in the structure of Fe-CS microparticles.

Study of the effectiveness of encapsulation and the controlled release of doxorubicin.

The results presented in fig. 2, a, demonstrate the positive effect of surface modification on doxorubicin EI. The presence of carboxyl groups (-COOH) on the surface of the microparticles significantly increases the encapsulation efficiency (more than 25%) compared to unmodified microparticles (Fe^0). Additionally, due to further surface modification (chitosan addition), the encapsulation efficiency was increased to 90%, which makes the Fe-CS system suitable for use as a drug carrier. This phenomenon can be explained as follows: because of the presence of positively charged amino groups, doxorubicin molecules enter into electrostatic interactions with the carboxyl groups of Fe-COOH microparticles. The binding of the components is enhanced by the appearance of hydrogen bonds [12].

In the Fe-CS system, DOX coupling was also carried out due to electrostatic interactions. Due to the large number of positively charged amino groups in the chitosan molecule, an increase in the loading capacity of negatively charged molecules occurs, which, in turn, leads to an increase in EI. Since the DOX molecule carries a positive charge, its attachment to Fe-CS microparticles takes place using a negative charged cross-linker (STP) in the form of a DOX-STP complex. Moreover, an increase in EI may be associated with an increase in the number of hydrogen bonds. The effect of pH on EI was confirmed.

The experiment showed a consistent decrease in EI with increasing pH values. For Fe-COOH microparticles with increasing pH from 3.3 to 5.5, EI decreased by 11%. With a stronger increase (from 3.3 to 7.4), the EI decreased by 18% (see fig. 2, a). This is due to the deprotonation of functional groups ($-NH^+_3$ and -COOH), which, in turn, leads to a change in their charges and weakening of electrostatic interactions.

In the Fe-CS system, a decrease in EI with an increase in pH value due to a decrease in the strength of electrostatic interactions between the functional groups of DOX, chitosan and STP was also observed. In this case, due to the presence of a hydrophilic coating (chitosan), an increase in the

number of hydrogen bonds occurs, which increases the stability of the conjugate.

In fig. 2, b, an assessment of the effect of doxorubicin concentration in the reaction mixture on the encapsulation efficiency is presented. In the case of Fe^0 microparticles, a stable decrease in EI was observed after its initial decrease. With a DOX concentration of 0.5 mg/ml, an equilibrium was reached between the adsorption-desorption processes (EI=5%), as evidenced by a further consistent decrease in EI with the increasing DOX concentration.

In the case of Fe-COOH and Fe-CS microparticles, a similar trend was observed, but, in contrast to Fe^0, a decrease was observed over the entire range of concentrations studied. As a result, the data obtained confirm the effect of surface modification on the encapsulation efficiency. Additionally, in the course of the experiment, the value of the loading capacity was determined, which amounted to 0.9 mg of doxorubicin per 1 mg of Fe-CS microparticles.

The assessment of the possibility of controlled release of doxorubicin from the Fe-CS system was

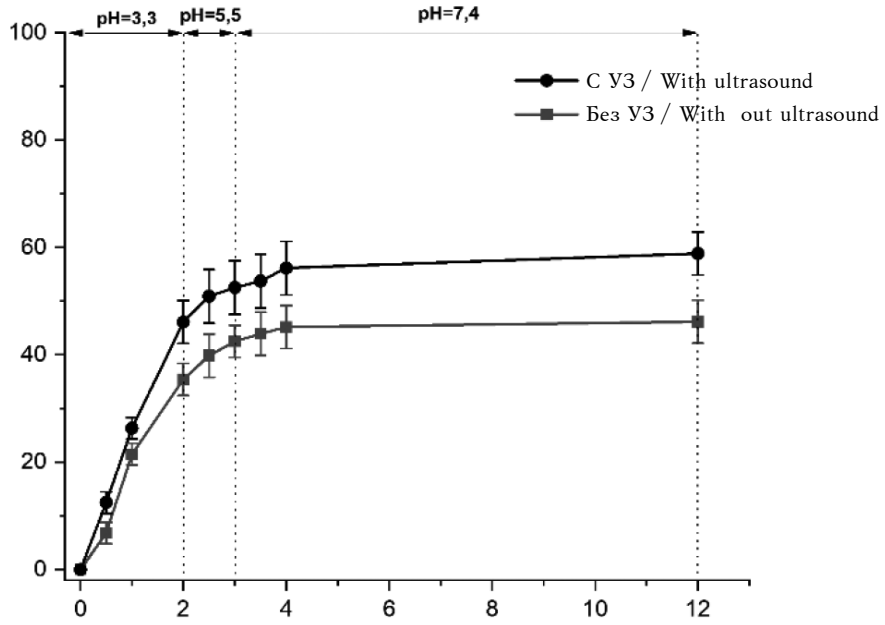

Fig. 3. Release of doxorubicin from conjugate Fe-CS / DOX along the OX axis – time, h; OY axis – quantity, %

Рис. 3. Высвобождение доксорубицина из конъюгата Fe-CS/DOX: по оси OX – время, ч; по оси OY – количество, %

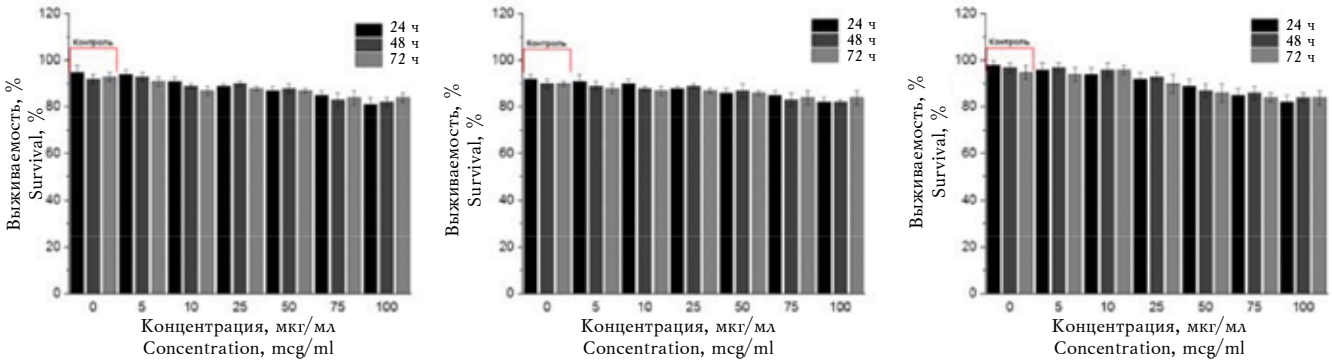

Fig. 4. HeLa cell line viability in presence of Fe^0 (*a*), Fe-COOH (*b*) and Fe-CS (*c*) microparticles depending on their concentration and the time of cell cultivation

Рис. 4. Выживаемость клеточной линии HeLa в присутствии микрочастиц Fe^0 (*a*), Fe-COOH (*b*) и Fe-CS (*c*) в зависимости от их концентрации и времени культивирования клеток

performed in an in vitro experiment under the influence of ultrasonic radiation with a successive change in pH values.

The results obtained in the course of the experiment (Fig. 3) confirm the effect of ultrasonic radiation on the rate of release of the therapeutic agent, regardless of the pH of the medium. This effect is due to the appearance of cavitation bubbles in the release medium when exposed to ultrasound. Their further destruction leads to a shear gradient, which, in turn, causes stretching and breaking of chemical bonds [25]. The attachment of system components, including the therapeutic agent, is due to electrostatic interactions, which are less durable than chemical bonds. As a result, gradual stretching leads to their destruction and accelerated release of doxorubicin. Thus, for the system under study, the possibility of controlled release of doxorubicin was confirmed.

Cytotoxicity study. The results of studying the cytotoxicity of microparticles are shown in fig.4.

The diagram shows that the values of cell viability in the presence of various concentrations of the microparticles under study do not differ from those observed in the control (values with a microparticle concentration of 0 μg/ml), which indicates the absence of independent cytotoxic properties of the developed DDS (in the absence of a loaded chemotherapeutic agent).

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Ди Мартино А., Власов С.С. – проведение практической части исследования, разработка концепции, дизайна, анализ и интерпретация данных. Гурьев А.М., Юсубов М.С., Постников П.С., Белоусов М.В. – разработка концепции, дизайна, проверка критически важного интеллектуального содержания, окончательное утверждение для публикации рукописи.

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Поступила в редакцию 03.05.2018
Подписана в печать 14.12.2018

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Received 03.05.2018
Accepted 14.12.2018