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Improving the Antitumor Effect of Doxorubicin in the Treatment of Eyeball and Orbital Tumors

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Abstract

Malignant tumors of the orbit are the main cause for 41–45.9% of orbital tumor, and they will threaten both the organ of vision and the life of the patient. In our opinion, improving the effectiveness of treatment of malignant tumors can be implemented in the following areas: a) immobilization of doxorubicin in synthetic polymeric materials, which will fill the tissue structures that were resected and reduce the percentage of tumor recurrence. b) the use of nanomaterials for the delivery of doxorubicin to tumor cells. To develop a hydrogel implant and nanoparticles, to study the diffusion kinetics of doxorubicin in a hydrogel implant and the ability of nanoparticles to transport doxorubicin. The developed gels based on acrylic acid (AAc) were obtained by radical polymerization of an aqueous solution of monomers (AAc and N, N-methylenebisacrylamide (MBA)) at a temperature of 70°C. Matrices based on polyvinyl formal (PVF) were obtained by treatment of polyvinyl alcohol (PVA) with formaldehyde in the presence of a strong acid. Experimental studies were performed on rabbits of the Chinchilla breed, weighing 2–3 kg, aged 5–6 months, which during the study were in the same conditions. We implanted the hybrid gel in the scleral sac; orbital tissue and in the ear tissue of rabbits: Evaluation of the response of soft tissues and bone structures to implant materials was carried out on the basis of analysis of changes in clinical and pathomorphological parameters was performed after 10, 30 and 60 days. Diffusion of doxorubicin was examined by using UV spectroscopy [spectrophotometer-fluorimeter DS-11 FX + (DeNovix, USA)], analyzing samples at regular intervals during the day at a temperature of 25° C. The concentration of active substances was determined by the normalized peak absorption of doxorubicin at 480 nm. The release kinetics of the antitumor drug doxorubicin were investigated by using a UV spectrometer “Specord M 40” (maximum absorption 480 nm). The developed hydrogel implant has good biocompatibility and germination of surrounding tissues in the structure of the implant, as well as the formation of a massive fibrous capsule around it. An important advantage of the implant is also the lack of its tendency to resorption. Moreover, the results showed that the diffusion kinetics of doxorubicin from a liquid-crosslinked hydrogel reaches a minimum therapeutic level within a few minutes, while in the case of a tightly crosslinked - after a few hours. It was also found that the liquid-crosslinked hydrogel adsorbs twice as much as the cytostatic - doxorubicin. The analysis of the research results approved that

the size of the nanoparticles is the main factor for improving drug delivery and penetration. Thus, nanoparticles with a diameter of less than 200 nm can penetrate into cells and are not removed from the circulatory system by macrophages, thereby prolonging their circulation in the body. About 10 nm. The developed hybrid hydrogel compositions have high mechanical strength, porosity, which provides 100% penetration of doxorubicin into experimental animal tissues. It was found that the kinetics of diffusion of drugs from liquid-crosslinked hydrogel reaches a minimum therapeutic level within a few minutes, whereas in the case of densely crosslinked hydrogel diffusion begins with a delay of several hours and the amount of drug released at equilibrium reaches much lower values (20–25%). The obtained preliminary experimental results allow us to conclude that our developed pathways for the delivery of drugs, in particular, doxorubicin to tumor cells will increase the effectiveness of antitumor therapy.

Keywords: eyeball and orbit tumors, doxorubicin, synthetic polymeric materials, nanomaterials

1. Introduction

Malignant tumors of the orbit represents the main cause for 41–45.9% of orbital tumors [1, 2] and they will threaten both the organ of vision and the life of the patient. According to some authors [2], recurrence of malignant tumors of the orbit within 5 years was observed in 36 of the 56 patients who were observed and recurrence of the tumor was observed in 64.3% of patients who died in subsequent years. As can be seen from the above data, the results of treatment of malignant tumors of the orbit are not satisfactory. In the analysis group of 56 patients with malignant tumors, only 36% had a 5-year survival.

The main method of treatment of orbit tumors is surgery, followed by radiotherapy and chemotherapy [3, 4].

However, surgical treatment of malignant tumors of the orbit leads to anatomical and functional damage. Independent use of radiation therapy does not lead to the desired result. In recent years, with the advent of new drugs and a deeper understanding of the theory of chemotherapy, the effectiveness of chemotherapy for malignant tumors has improved significantly. The clinician always has the task of creating a sufficient concentration of the drug in the tumor area in order to obtain a therapeutic result and at the same time minimize the load on healthy cells during the local tumor process.

In our opinion, increasing the effectiveness of treatment of such type of tumor can be detected by delivering high concentration of doxorubicin in the tumor in the following ways;

- a. Immobilization of doxorubicin in synthetic polymeric materials, which will fill the tissue structures that were resected, and long-term withdrawal of the drug will significantly reduce the recurrence rate of tumors.
- b. Use of nanomaterials for delivery of doxorubicin to tumor cells.

To implement the first task, we needed to develop non-biological implants with a porous hollow structure that are capable of biointegration with the surrounding orbital tissues.

2. To develop non-biological implants and study the soft tissue response to it

Such capabilities are possessed by hydrogels - spatially crosslinked hydrophilic polymers that have been successfully used for several decades as materials for tissue engineering and plastic surgery, means for targeted transport of drugs, optical and analytical sensors, matrices for biological research [5], etc. Abnormally high compared with solid polymers, the biocompatibility of gels with high equilibrium water content, primarily due to the similarity of their 3D structure with the extracellular matrix [6]. Achieving a significant improvement in the physicochemical and operational parameters of gels seems to be possible by obtaining a hybrid hydrogel material based on polyvinyl alcohol and acrylic hydrogel, which was the subject of one of the studies performed by the authors [7].

Material and methods. Gels based on acrylic acid (AAc) were obtained by radical polymerization of an aqueous solution of monomers (AAc and N, N'-methylenebisacrylamide (MBA)) at a temperature of 70°C. Matrices based on polyvinylformal (PVF) were obtained by treating polyvinyl alcohol (PVA) with formaldehyde in the presence of a strong acid.

Experimental studies were conducted on the basis of the vivarium of the State Institution "The Filatov Institute of Eye Diseases and Tissue Therapy of the NAMS of Ukraine". Experimental studies were performed on rabbits of the Chinchilla breed, weighing 2–3 kg, aged 5–6 months, which during the study were in the same living conditions. All experimental studies were conducted in compliance with ethical standards provided by the international principles of the European Convention on the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (Strasbourg, 1986) and the norms of biomedical ethics approved by the First National Congress of Bioethics of Ukraine), as well as the law of Ukraine №3447-IV "On protection of animals from cruel treatment" (Kyiv, 2006).

To study the reaction of the soft tissues of the orbit and auricle, we used an implant hybrid hydrogel developed at the Ovcharenko Institute of Biocolloid Chemistry of the National Academy of Sciences of Ukraine in Kyiv. We implanted a hybrid hydrogel in a scleral sac; orbital tissue and in the ear tissue of rabbits.

We implanted a hybrid hydrogel implant into the scleral sac, parabulbar tissue of the eyeball and auricle of a rabbit/.

Surgical interventions were performed under general anesthesia (at the rate of 1 ml of 0.1% sodium thiopental solution per 1 kg of rabbit body weight intramuscularly). Evaluation of the response of soft tissues and bone structures to implant materials was carried out on the basis of analysis of changes in clinical and pathomorphological parameters was performed after 10, 30 and 60 days. Evaluation of the studied clinical signs (swelling of the tissues of the orbit, cheeks, auricle, the condition of the sutures, the presence of secretions) was performed on the 2nd, 5th, 10th and then every five days. Pathohistological evaluation of oculoorbital tissues, orbit and auricle tissue was performed after 10, 30 and 60 days.

Results and discussion. Analysis of scanning electron micrographs of the hybrid hydrogel material showed that its structure is characterized by a well-developed system of connected pores smaller than 1 mm, as well as the presence of pores with a diameter of several hundred micrometers. It should also be noted that the pore walls have a porous structure with an approximate pore diameter of 10 µm and a wall thickness of several micrometers. Thus, due to partial squeezing of the gel-forming composition from the pore space of the spongy polymer matrix based on the PVF, it was possible to prevent clogging of open and combined transport pores,

Figure 1.
Appearance of a hybrid hydrogel implant.

which allowed to ensure high permeability of hybrid material to gases, liquids and biological tissues (**Figure 1**).

In general modification of the PVF with acrylic hydrogel decreased the size of pores inside the walls for all hydrogel systems and also fill the larger pores with diameters approx. 500 μm . The synthesized polymer systems belong to porous materials with pore diameters from a few micrometers to a few hundred micrometers. Nanopores have not been found in synthesized systems [8].

Experimental studies in rabbits showed that in the first 5 days after implantation of the hybrid hydrogel in the scleral sac, orbit and auricle, all animals had swelling of the postoperative suture and adjacent conjunctiva, as well as a slight serous-secretion from the conjunctival cavity. After five days, there was a decrease in edema and discharge from the conjunctival cavity and lasted for 8–10 days. It is important to note that when examining the postoperative wound of the skin and conjunctiva in the first days and in the following days with the implantation of a hybrid hydrogel, wound healing was the primary tension.

The obtained satisfactory result of clinical evaluation of soft tissues of the orbit and auricle to the implantation of a hybrid hydrogel, we found it appropriate to assess the response of cellular structures to the implant, the presence of germination of surrounding tissues in its structure and propensity to resorption.

Pathohistological studies showed that on the 10th day after the implantation of the hybrid hydrogel into the scleral sac, inflammatory infiltration of the sclera was noted in the site of the implant location. However, no inflammation was noted in the scleral portions distant from the implant.

In the infiltrate, in addition to lymphocytes, there is a fairly large number of eosinophilic leukocytes (**Figure 2a**).

An important requirement for the implant material is the ability to germinate the surrounding tissue structure of the implant, as well as its tendency to resorption. Therefore, it was expedient for us to study the influence of the surrounding biological tissues on the mesh-like structure of the implant (**Figure 2b**).

In **Figure 2b** it can be noted that the structure of the implant is preserved, and along the crossbars there is the formation of delicate bundles of collagen fibers, which are slightly infiltrated by lymphocytes. The composition of “cellular” structures is absent, possibly as a result of histological processing. We did not observe any changes on the side of the orbit walls.

In clinical practice, there is a need to fill the soft tissues after removal of tumors of the orbit, eyelids and oculo-orbital area. In this regard, we found it appropriate to study the relationship of the implant - a hybrid hydrogel with soft tissues and cartilaginous structures. Pathohistological studies have shown that when the implant is placed in the tissues of the auricle after 10 days, a delicate fibrous tissue is formed around it, which is infiltrated by inflammatory cells. At the same time the

Figure 2.
A and b. the tenth day after implantation of the hybrid hydrogel in the scleral sac; a - (1-implant; 2- inflammatory scleral infiltration) (hematoxylin–eosin; x 120), b - (1-implant; 2- fibrous capsule) (hematoxylin–eosin; x 70).

Figure 3.
Thirty days after implantation of the hybrid hydrogel in the scleral sac.

weak basophilic maintenance of “cellular” structures remains, and on partitions the initial phenomena of fibrotization are noted.

Thus, the assessment of clinical signs and the results of histopathological examinations after implant placement - a hybrid hydrogel in the scleral sac and tissues of the rabbit auricle allowed to draw preliminary conclusions, which were that within 8–10 days there was an inflammatory reaction from the tissues of the orbit and auricle, especially the first 5 days. It is important to note that in no case did we notice the implant being exposed and the wound healing was the primary tension. Pathohistological studies showed that around the implant, both in the scleral sac and in the tissues of the auricle there are all signs of inflammation (lymphoid and leukocyte infiltration, etc.), the presence of signs of germination surrounding the tissue structure of the implant and its tendency to resorption. Having obtained preliminary data, it was expedient for us to study the nature of the interaction of the implant - a hybrid hydrogel with the surrounding tissues in the longer term. Therefore, we decided to assess the nature of histopathological changes after 30 days.

When placing the implant in the scleral sac after 30 days, inflammatory phenomena around it is absent, but there was the formation of a fibrous capsule with the spread of fibrous tissue on the partitions of “cellular” structures. The fibrous layers are quite rough and do not contain inflammatory elements (**Figure 3**). Up to 30 days, the content of “cellular” structures was determined by the wall and in small quantities.

Formation of a fibrous capsule and massive growth of fibrous tissue along the partitions of “cellular” structures (1-implant; 2- growth of fibrous tissue along the partitions of “cellular” structures) (Hematoxylin–eosin; x 180).

A similar pathohistological picture was observed after 30 days of the implantation of a hybrid hydrogel in the tissues of the auricle, which consisted in fibrotization of the walls of the “honeycomb” structures of the implant without signs of inflammation. It should be noted that after 60 days we did not notice signs of inflammation around the implant except for the formation of a fibrous capsule.

The obtained experimental studies, which study the nature of the reaction of soft tissue bone structures of rabbits to the implantation of a hybrid hydrogel in the scleral sac and auricle tissue, allowed to answer a number of questions that arise in the development of implant materials.

The first and most important requirement for implants is their biocompatibility. As our studies showed, in all experimental rabbits there was a moderate inflammatory reaction, which disappeared by 8–10 days. It is important to note that in all groups of animals studied wound healing was the primary tension, and therefore in no case was the exposure of the implant, which indicates a very important positive indicator for implants. Pathohistological studies showed that up to 10 days there are final inflammatory phenomena close to the implant in the form of lymphoid and leukocyte infiltration, while in more remote areas relative to the implant, they were absent. The second important indicator we noted is the germination of the surrounding tissues in the implant structure and the formation of a delicate fibrous capsule by the tenth day after its implantation (**Figure 2b**), and by the 30th day - the formation of a massive fibrous capsule (**Figure 3**). The third important advantage of the implant is the lack of its tendency to resorption, which is very important to obtain a stable clinical result. It is also important to note that we did not observe changes in the bone structures of the orbit and in the cartilaginous plate of the rabbit auricle when placing the implant in the soft structures of the orbit and auricle.

Our in vivo experimental studies demonstrated that the high biocompatibility of the hybrid highly porous material based on polyvinylform developed by them, the lack of resorption and the ability to germinate the surrounding biological tissues. This indicates the high prospects of the developed material and provides grounds for further research aimed at improving its performance.

3. Influence of the structure of hydrogels hydrogel implants on dynamics of deposition and diffusion of doxorubicin

It should be noted that it is important to prevent the recurrence of the malignancy after its removal, so it is important that the implant materials contain antitumor drugs.

The hybrid hydrogels used are spatially crosslinked hydrophilic polymers that are characterized by a unique combination of properties such as high hydrophilicity, softness, flexibility and strength, as well as unique biocompatibility [9]. Due to their ability to absorb significant amounts of water and biological fluids, porosity and elasticity, they more than any other synthetic biomaterials resemble human tissues and have been successfully used for decades as a means of targeted transport and prolonged drug release [10], biosensors [11], anti-burn and hemostatic dressings [12], materials for tissue engineering and plastic surgery [13], etc. Given the possibility of using the proposed hybrid hydrogels in the creation of implants that will have the ability to deposit drugs, primarily antimicrobial and antitumor, we thought it appropriate to study the diffusion properties of hybrid hydrogels with different porosity and with immobilized drugs, in particular drugs.

Materials and methods. The research was conducted in the department of functional hydrogels of the Ovcharenko Institute of Biocolloid Chemistry, NAS of Ukraine using the following substances and materials:

Reagents for the synthesis of hydrogels: Polyvinyl alcohol (PVA) (AppliChem GmbH, 98%; 72 kDa); formaldehyde (LAB-SCAN, 37%); concentrated sulfuric acid (AAc); Triton X-100 (AppliChem GmbH); acrylic acid (99%, Sinbias); ammonium persulfate (PSA) (Thermo Fisher, 98%); N, N'-methylenebisacrylamide (Merck) was used without further purification.

Medicinal product: Doxorubicin "Ebeve" - concentrate for solution for infusion containing 2 mg/ml For saturation with pharmaceuticals, hydrogels of different densities were obtained, varying the mass fraction of PVA. The content of PVA in the liquid-crosslinked gel was 7.1 wt. %, and in a tightly crosslinked gel - 8.0%. For the manufacture of a hybrid hydrogel based on pre-synthesized polyvinylformal and acrylic acid, 0.6 g of PVF was placed in a medical syringe with a capacity of 10 ml and impregnated with a solution containing 0.6 ml of AAc, 0.2 ml of 3% MBA solution and 5.25 ml of 40% solution of PSA. After impregnation, 4.5 ml of liquid was squeezed out and the resulting polymerization composition was placed in an oven at 40° C for 1 hour.

Spectral analysis (FTIR) of wet samples was performed using a spectrometer Spectrum BX FT-IR (Perkin Elmer). The spectra were recorded using the method of disturbed total internal reflection (internal reflection spectroscopy) in the spectral range 4000–550 cm⁻¹ with a resolution of 2 cm⁻¹. Each spectrum was recorded 8 times to prevent accidental artifacts.

The porosity of the samples was determined by gravimetric method according to the formula:

$$P = \left(1 - \frac{m}{V \cdot \rho} \right) \times 100\% \quad (1)$$

where P is the total porosity [%], m is the dry weight of the sample, V is the volume of the dry sample, ρ is the density of the material used [14]. When measuring the pore size, an optical microscope SIGETA MB 140 LED Mono was used to study the drugs in transmitted, reflected and mixed light. Toup View 3.5 was used to process statistical data and video images of the microscope.

Detailed information on the structure of the pore space of polymer systems was obtained from the analysis of microphotographs taken using a scanning electron microscope JSM-6060 LA (JEOL, Japan) with a resolution of 4 nm. The polymer samples were freeze-dried in a sublimation unit UZV-10 (Kharkov, Ukraine), attached to standard holders with a double-sided conductive film and covered with a layer of Au/Pd with a thickness of 25 nm in the ion-spray unit Gatan 682 Precision Patching and Coating System Gatan, USA).

The kinetics of swelling of the samples of the proposed hydrogels was studied at a temperature of 25°C in distilled water and saline (0.9% aqueous sodium chloride solution), determining the degree of swelling of Q samples weighing 23.8–27.0 mg by gravimetric method according to the formula: $Q_t = (m_t - m_d)/m_d$, where Q_t and m_t are the degree of swelling and the mass of the swollen sample in a certain time interval, m_d is the initial mass of the dry sample [15, 16].

Diffusion of doxorubicin in hybrid hydrogels was studied as follows. Samples of dry hydrogels in the form of cylinders with a diameter of 12 mm and a weight of 50 mg (height varied from 5 mm to 8 mm depending on the composition of the hydrogel) for saturation were placed in 0.02% doxorubicin solution for

18 hours at a temperature of 25°C. After saturation, the mass of the drug was calculated taking into account changes in the initial concentration. Excess fluid was squeezed from the swollen samples using a disposable medical syringe, the squeezed samples were weighed and placed in vials of 20 ml of saline. Diffusion of doxorubicin was examined by UV spectroscopy using a spectrophotometer-fluorimeter DS-11 FX + (DeNovix, USA), analyzing the samples at certain intervals during the day at a temperature of 25°C and periodic stirring. The concentration of active substances was determined by the normalized peak absorption of doxorubicin at 480 nm.

Results and discussion. IR spectroscopy. Based on the obtained IR spectra, the functional groups of porous matrices based on PVF were characterized (**Figure 4**). Wide and intense peaks in the region of about $3362\text{--}3382\text{ cm}^{-1}$ can be attributed to the valence vibrations of hydroxyl groups. The expansion of these absorption bands is explained by the hydrogen bonds that the OH groups join. Bands at 1007 cm^{-1} on the spectrum of the matrix based on PVF are also characteristic of the valence vibrations of the hydroxyl group of primary alcohols C OH.

According to the calculations performed by formula (1), the high-density crosslinked hydrogel had a porosity of 91.8%, and the low-density crosslinked hydrogel - 95.0%. The calculated porosity of the hydrogel composition with acrylic acid was 85.9%.

According to light-optical and electron microscopy, the obtained hydrogels had a heterogeneous multilevel porous architecture. That is, the pores of the highest level were also formed from porous structures that were about two rows smaller. According to the calculations, the pores of the highest level had a diameter of $120\text{--}180\text{ }\mu\text{m}$ in a high-density crosslinked hydrogel (**Figure 5a**) and $460\text{--}670\text{ }\mu\text{m}$ in a low-density crosslinked hydrogel (**Figure 5b**). The pore size in the hybrid hydrogel with acrylic acid varied from $200\text{ }\mu\text{m}$ (**Figure 6a**) to $590\text{ }\mu\text{m}$ [15].

For small pores of the lowest level, which form the substructure of the walls of large pores (**Figure 6a**), regardless of the density of the hydrogel, their diameter was $3\text{--}5\text{ }\mu\text{m}$ (**Figure 6b**).

The porosity and pore size of the implant material play a significant role in tissue regeneration, so these parameters are widely studied and discussed in numerous studies. The porous structure of the matrix is necessary for tissue regeneration, because it depends on the adhesion, migration and proliferation of cells, as well as the diffusion of nutrients, oxygen and metabolites. It has been found that large pores provide nutrient delivery and removal of metabolic products, while small pores provide a larger surface area for cell adhesion [10, 17]. It should be noted that the influence of pore architecture on the behavior of cells also depends on

Figure 4.
IR spectrum of a porous matrix based on PVF.

Figure 5.
A and b. porous structure of high-density crosslinked (a) and low-density cross-linked (b) PVF hydrogels according to the processing of microscopic images 40x.

Figure 6.
A and b. electron microscopic image of the pore architecture of PVF hydrogels: A - pores of the highest level, b - pores of the substructural level.

their nature. *In vitro* experiments have shown that 380–405 μm pores are best for chondrocytes and osteoblasts, while fibroblasts are prone to proliferation in smaller diameter pores (186–200 μm) [18]. Such preferences may be explained by the fact that, although large pores improve the diffusion of nutrients and oxygen, fibroblasts tend to cling to the substrate with smaller pores, as this increases the area of specific contact [17]. According to *in vitro* studies, greater porosity is accompanied by increased cell migration and infiltration [19, 20]. At the same time, *in vivo* it can be the cause of protein leaching [17, 21]. Also an important characteristic is the interporous connection, which depends not only on the diffusion properties of the matrix in relation to nutrients and oxygen, but also the possibility of ingrowth of newly formed vessels [21].

Based on this, it can be argued that our proposed hydrogels are able to accumulate and transport through a system of small pores of various metabolites and drugs, as well as serve as a matrix for attachment and migration of different cell types that ensure regeneration processes in biological tissues.

Kinetics of hydrogel swelling. From the analysis of the kinetics of swelling of hydrogels of different structure it can be concluded that all samples of hydrogels in water reach an equilibrium state of swelling in the first 30 minutes. In saline, the degree of swelling of the hydrogels was slightly lower (approximately 16%), but this had little effect on the high rate of swelling. Only in the composite hydrogel with acrylic acid, the equilibrium state in saline was reached within 3 hours. This pattern is inherent in hydrogels in general and is explained by a decrease in ionic osmotic pressure, which causes swelling of hydrogels, with increasing ionic strength of the solution. The obtained results were used to determine the period of saturation of hydrogel samples with drugs.

3.1 Diffusion of doxorubicin from hydrogels

The diffusion kinetics of doxorubicin are shown in **Figure 7**. The low-density cross-linked hydrogel sorbs twice as much doxorubicin as the high-density cross-linked hydrogel, possibly due to the absence of steric obstacles to penetration of the porous hydrogel structure by a large drug molecule (molecular weight is 544 g/mol). In *in vitro* experiments, the former hydrogel provided a 3–4-fold greater drug concentration in the environment compared to the latter hydrogel (**Figure 7**). The latter hydrogel, however, allows for a smoother and more prolonged drug release profile and therefore it is advisable to use for implants with a prolonged drug effect, while low-density crosslinked hydrogel - for urgent release of a shock dose of cytostatic preparation.

Hybrid hydrogels based on PVF and incorporated poly-AAc have a much greater - about an order of magnitude - the ability to deposit doxorubicin (compared to PVF). This effect may be due to the formation of ionic bonds between the active -COOH groups present in the hybrid hydrogel and the amine groups present in the structure of doxorubicin. The slow hydrolysis of these ionic bonds explains the prolonged (several days) release of doxorubicin from hybrid hydrogels (**Figure 8**).

This prolonged ability of hybrid hydrogel implants will facilitate their use for the deposition of antitumor drugs and maintain their effective concentration in the pathological focus.

Thus, studies have shown that the kinetics of diffusion of drugs from liquid-crosslinked hydrogel reaches a minimum therapeutic level within a few minutes, whereas in the case of densely crosslinked hydrogel diffusion begins with a delay of

Figure 7. Diffusion of doxorubicin from low-density crosslinked (1, 2) and high-density cross-linked (3, 4) hydrogel during the day (— - release into solution; ----- - release percentage).

Figure 8. Diffusion of doxorubicin from saturated samples of composite hydrogel containing polyacrylic acid during the week; A-concentration, µg/ml; B-percent release from sorbed.

several hours and the amount of drug released at equilibrium is much lower. Values (20–25%). It has also been found that the liquid crosslinked hydrogel absorbs twice as much cytostatics as doxorubicin, which may be due to the lack of steric barriers to the penetration of the bulk molecule of doxorubicin (molecular weight 544 g/mol) into its porous structure. This hydrogel provides *in vitro* experiments 3–4 times higher concentration in the environment compared to densely crosslinked polymer, and also provides a smoother, prolonged release of the drug.

It is important to note that the main factor in antitumor therapy is the temporary parameter of tissue saturation with drugs, in this regard, we continue to study in this direction. However, this direction of antitumor therapy is accompanied by surgery.

4. Nanoparticles, possible way of delivery of doxorubicin to tumor cells

Noteworthy is the delivery of doxorubicin to tumor cells through the use of nanomaterials [22].

In recent years, researchers have focused on the so-called “smart” therapeutic systems that are able to respond to minor changes in their environment by a sharp change in their physicochemical (primarily diffusion) characteristics [23]. The greatest attention is paid to thermo- and pH-sensitive hydrogels, which under the influence of minor, physiologically acceptable changes in temperature or pH are capable of controlled mass release of drugs, in particular, anticancer [21].

Even, doxorubicin is one of the most effective and widely used drugs against a wide range of cancers. But, its clinical use in parenteral administration is accompanied by such side effects as high cardiac toxicity and myelosuppression. The most serious long-term adverse effect of doxorubicin therapy is irreversible cardiomyopathy, which is based on the total cumulative dose [22]. In one clinical study, ~4% of patients receiving dosages of 500–550 mg/m² developed congestive heart failure, 18% with dosages of 551–600 mg/m², and 36% with cumulative dosages higher than 601 mg/m² [24]. This necessitates the development of new therapeutic systems for the transport of doxorubicin, increasing its therapeutic efficacy and minimizing side effects. Ideally, therapeutic transport systems of doxorubicin should inhibit the release of the drug in plasma and release it only after reaching the tumor site by actively targeting tumor cells through endocytosis.

As a trigger for targeting the therapeutic transport system of doxorubicin can be used a significant difference in the pH of plasma (pH = 7.4) and the microenvironment of the tumor (pH = 6.5) and lysosomes (pH = 4.8–5) [6, 9, 21]. In particular, this determines the prospects for the use of pH-sensitive hydrogels for controlled transport of anticancer drugs, primarily doxorubicin.

However, nanogels that are sensitive to changes in both pH and temperature, such as, for example, hydrogel copolymers synthesized by us based on N-isopropylacrylamide (NIPAm) and acrylic acid (AAc), seem to be especially promising.

The synthesis of nanogels based on NIPA was described in detail earlier [25, 26]. N-isopropylacrylamide, NIPAm (Sigma-Aldrich, 97%) was recrystallized from hexane and dried under vacuum; N,N'-methylenebisacrylamide (MBA) (Merck, 98%), acrylic acid (AAc) was purified by distillation and subsequent fractional distillation, potassium persulphate, PSP (Sigma 98%) were used without additional purification, as well as sodium dodecylsulphate (SDS), polyethylenimine (Sigma-Aldrich MM 2000 Da) and iron salts (FeSO₄ and FeCl₃) used in magnetite synthesis. Briefly, 2,3 g of NIPAm, 0,0393 g of MBA, 0,1124 g of SDS, 5 mL of magnetite suspension, 0,115 g of AAc and 135 g of water were placed in the beaker.

After that the beaker was set on a magnetic stirrer to dissolve the reagents at room temperature. At the end of the mixing the solution in the beaker underwent purification with argon for 2 minutes. It was then transferred into the glass reactor equipped with a stirrer and thermometer. The reactor was placed in a water bath with that was maintained at constant temperature. The synthesis was carried out at 68–70°C. When the reactor temperature reaches these temperature, 10 mL of 0,93% solution of PSP in water was added. The mixing rate was 500 rotations per minute. The duration of the synthesis took additional 6 hours. The release kinetics of the antitumor drug doxorubicin were investigated using a UV spectrometer “Specord M 40” (maximum absorption 480 nm).

The size of the polymer carriers (transport systems) of anticancer drugs is of great importance, since nanoparticles with a diameter of less than 200 nm, on the one hand, are able to penetrate into cells, in particular, affected cells, and on the other hand, they are not captured by macrophages, which contributes to an increase in the duration of their circulation in the body. As can be seen from the Electronic Microphotographs (TEMs) shown in **Figure 9**, the synthesized nanogels are characterized by uniformity of shape and size and have an average diameter of about 100 nm. At magnification (see **Figure 9**, box), you can see incorporated into the nanopherogel nanoparticles of magnetite with a size of about 10 nm.

The obtained images correlate well with the results of dynamic light scattering measurements. It is shown that the average size of the synthesized hydrogels based on NIPAm and AAc is about 100–200 nm and depends on the temperature and pH value, as well as the value of the zeta potential of nanoparticles. Synthesized copolymer nanogels based on NIPAm and AAc combine thermo- and pH-sensitivity. When heated above the LCST (lower critical solution temperature, equal for NIPAm to a temperature of 32–34°C) and when the environment is acidified, the diameter of the nanoparticles decreases. Thus, it was found that the average size of nanoparticles of copolymer hydrogels based on NIPAA and AAc when heated from 25 to 50°C decreases by 3–5 times, which is a consequence of thermo-induced phase transition from swollen to collapsed state of the hydrogel (**Figure 10a and b**).

At the same time, in the acidic region, the pH of nanopherogels is about 10 nm, while in an alkaline environment increases by about an order of magnitude. Note that these processes are reversible and further cooling of the nanogels (as well as an

Figure 9. Microphotographs (TEM) of synthesized nano(ferro)gels based on NIPAm - AAc copolymer with incorporated magnetite.

Figure 10.
A and b. size distribution of nano(ferro)gels based on NIPAm – AAc copolymer with incorporated magnetite depending on: A - temperature at 50°C (1), 37°C (2) and 25°C (3); b - acidity of the medium at pH = 1.1 (1) and pH = 12.0 (2).

Figure 11.
A and b. size distribution (a) and zeta potential (b) of nano(ferro)gels based on NIPAm- AAc copolymer with incorporated magnetite and doxorubicin at temperatures of 25°C (1) 37°C (2) and 50°C (3).

increase in pH) leads to an increase in their size to the original values. This behavior of nanosized hydrogel matrices creates the preconditions for spontaneous targeted release of incorporated antitumor drugs, primarily doxorubicin when heated in a temperature-acceptable range, for example, in drug hyperthermia or in contact with affected cells, which are characterized by acidic pH.

Zeta potential as a function of the surface charge of a substance in a liquid is an excellent characteristic of electrostatic repulsion between particles. Zeta potential is usually used to predict and control the stability of the dispersion. Moreover, the characteristics of the solid–liquid interface can have a strong effect, in particular on adhesion, flotation, and in more concentrated systems on the rheological behavior of the system.

Thus, it shown that an increase in temperature in the range of 25°C to 37°C, up to 50°C leads to an increase (in absolute value) of the zeta potential and a decrease in the size of ferrogel nanoparticles, which indicates an increase in aggregate stability of the corresponding colloidal systems. This pattern can be explained by the rupture of intermolecular hydrogen bonds that promote aggregation, with increasing Brownian motion when heating NIPAm macromolecules.

Thermo- and pH-sensitive copolymer hydrogels based on NIPAm and AAc with incorporated magnetite and cytostatic doxorubicin were also characterized using a Zetasizer Nano ZS (Malvern Instruments) zeta-seiser. It was demonstrated (**Figure 11a** and **b**) that as the temperature increases (in the physiologically acceptable range), the size of nanoparticles decreases (to about 50 nm) and

the zeta potential increases, which indicates an increase in the aggregate stability of nanosuspensions.

Taking in the account medical field of application of synthesized nano(ferro) gels, an extremely important problem is their washing from the unreacted monomers and other toxic pristine materials since the gelation reaction never proceeds with 100% conversion. Washing of medical nanogels from monomer residues and unreacted initiators is carried out by long-term extraction (for 4–10 days) with a suitable solvent (preferably water) with its repeated replacement.

As it can be seen from **Figure 12**, the immediately after synthesis, the concentration of NIPAm monomer significantly exceeds the maximum allowable level. After washing 5 times, the concentration of monomer decreases 50 times, and after seven times - more than 500. Analyzing the size distribution spectra of the crude nanogel samples and the corresponding samples after washing, obtained by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments), we can conclude that due to washing using diafiltration the average size of nanoparticles increases slightly, which, in our opinion, is associated with the leaching of surface-active sodium dodecyl sulfate, which prevented the aggregation of nanoparticles [27].

It can be seen from **Figure 12**, the concentration of NIPAm monomer significantly exceeds the maximum allowable level immediately after the synthesis. While, after washing 5 times, the concentration of monomer decreases 50 times, and after seven times – drop down more than 500 times.

In the case of macrogels, the temperature of their phase transition between the swollen and collapsed state (which determines the possibility of controlling their physicochemical properties, primarily diffusion) is determined gravimetrically, which is almost impossible in the case of nanogels. However, it has been demonstrated that the phase transition temperature can be determined no less accurately by measuring light transmission. As can be seen from **Figure 13**, ($\square$), at temperatures below 30°C hydrogels are in the expanded conformation, while when heated above 32°C (lower critical solution temperature, LCST) there is a phase transition to a compact collapsed state due to the destruction of hydrogen bonds between molecules water and hydrophilic amide groups of NIPAm caused by Brownian motion, as well as the strengthening of hydrophobic interactions of isopropyl groups of NIPAm. As a result, there was a sharp decrease in the light transmission of dispersions, and the phase transition temperature for nanopherogels was about 35°C. A small increase in the magnitude of the phase transition from the inherent NIPAm temperature of 32°C is explained by its copolymerization with hydrophilic acrylic acid. Due to the established effect of increasing the NIPA phase transition temperature when copolymerized with a more hydrophilic monomer (eg acrylamide or AAc) and decreasing the NIPAm phase transition temperature when copolymerized with a more hydrophobic monomer (eg acrylonitrile), therapeutic systems based

Figure 12.

A and b. reduction of absorption (a) of monomeric NIPAm in the UV region (1-after washing; 2-immediately after synthesis); change in the concentration of NIPAm depending on the number of washes (b).

Figure 13.
The effect of temperature on light transmission for crude (□) and purified (◇) nano(ferro)gels based on NIPAm and AAc with incorporated magnetite at pH = 7.

Figure 14.
Kinetics of release of doxorubicin from nano(ferro)gels based on NIPAm and AAc with incorporated magnetite at pH = 7 (nano(ferro)gel was saturated with a solution of the drug with a concentration of $2.5 \times 10^{-2}\%$).

on delivery and controlled release at the desired temperature of various drugs, in particular doxorubicin.

The analysis of **Figure 14** shows that at 25°C the release of the antitumor drug doxorubicin is completed after 30–40 min, whereas heating above the LCST causes spontaneous release of the drug from the collapsed hydrogel. Thus, the synthesized nano (ferro) gels based on NIPAm, AAc and magnetite due to their unique properties are a promising material for the creation of therapeutic systems of targeted delivery and controlled release of drugs, in particular, in drug hyperthermia.

Incorporation of pre-synthesized nanosized magnetite into nanogels allows to give the corresponding nanophero gels magnetically controlled properties, namely - the possibility of targeted localization under the influence of a constant magnetic field of anticancer drug carriers in close proximity to the target organ, which is extremely important. Means and the need to minimize their overall impact on the body. Thus, the incorporation into the composition of hydrogel matrices of nano-sized magnetite provides the possibility of targeted localization of the developed therapeutic systems in close proximity to the target organ by applying a constant low-intensity magnetic field with subsequent controlled release of incorporated drugs (primarily, cancer-free - or low-intensity alternating magnetic field).

For nano(ferro)gels on the base of NIPAm, biological studies were performed that showed that magnetic hydrogels with a magnetite content of up to 10% are not toxic to PTP cells (primary swine testicle) (**Figure 15a**). Moreover, upon contact of the original matrix with the cells, it was found that their activity tends to increase compared to the activity of control intact cells. Therefore, the result allows us to

Figure 15.

a and b. Cytotoxicity of nano(ferro)gels based on NIPAm for PTP cells (a) and HEP-2 cells (b).

consider nano(ferro)gels based on NIPAm hydrogels, suitable for the development of hyperthermia of cancer cells, targeted delivery and controlled release of drugs, as well as objects for cell growth. Similar results were obtained in the study of cytotoxicity for HEP-2 cells (epidermal carcinoma of larynx) (**Figure 15b**).

For copolymer ferrogels with a 95% NIPAA content, biological studies performed showed that magnetic hydrogels with a magnetite content of up to 10% are not toxic to PTP cells (**Figure 15a**). Moreover, upon contact of the original matrix with the cells, it was found that their activity tends to increase compared to the activity of control intact cells. Therefore, the result allows us to consider ferrogels based on copolymer hydrogels, which contain 95% NIPAA and 5% AA, suitable for the development of hyperthermia of cancer cells, targeted delivery and controlled release of drugs, as well as objects for cell growth. Similar results were obtained in the study of cytotoxicity for HEP-2 cells (**Figure 15b**).

5. Conclusion

From the above points, it can be concluded that the hydrogel implant developed by us will allow to fill soft tissue structures quite effectively during tissue resection. However, this will partially solve the problem. The clinician always faces such an important task as to avoid tumor recurrence. Immobilization and diffusion of doxorubicin into the implant showed that the kinetics of diffusion of the drug from the liquid-crosslinked hydrogel reaches a minimum therapeutic level within a few minutes, whereas in the case of densely crosslinked hydrogel diffusion begins with a delay of several hours and the amount is released. Much smaller values (20–25%). It is also shown that the tightly crosslinked hydrogel has a higher ability to deposit doxorubicin, and therefore, it is advisable to use for implants with a prolonged antibacterial effect, while the liquid crosslinked hydrogel - for the immediate release of a shock dose of antiseptic. It is important to note that the liquid-crosslinked hydrogel absorbs twice as much cytostatics as doxorubicin, which may be due to the lack of steric barriers to the penetration of the bulk molecule of doxorubicin (molecular weight 544 g/mol) into its porous structure. This hydrogel provides in vitro experiments 3–4 times higher concentration in the environment compared to densely crosslinked polymer, and also provides a smoother, prolonged release of the drug.

The obtained preliminary experimental results allow us to conclude that our developed pathways for the delivery of drugs, in particular, doxorubicin to tumor cells will increase the effectiveness of antitumor therapy. We are faced with many questions that we will implement in further research.

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