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Noninvasive control of rhodamine-loaded capsules distribution *in vivo*

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ABSTRACT

Using fluorescence spectroscopy system with the fibre-optical probe, we investigated the dynamics of propagation and circulation in the microcirculatory system of experimental nanocapsules fluorescent-labelled (rhodamine TRITC) nanocapsules. The studies were carried out in clinically healthy Wistar rats. The model animals were divided into control group, and the group received injections of the nanocapsules. The fluorescent measurements conducted transcutaneously on the thigh surface. The administration of the preparation with the rhodamine concentration of 5 mg/kg of animal weight resulted in a twofold increase of fluorescence intensity by reference to the baseline level. As a result of the study, it was concluded that fluorescence spectroscopy can be used for transdermal measurements of the rhodamine-loaded capsules in vivo.

Keywords: nanocomposite polymeric capsules, fluorescent labelled particles, rhodamine TRITC, drug delivery, fluorescence spectroscopy, blood microcirculation, optical measurements in vivo

1. INTRODUCTION

Conventional control of administrated substance distribution in animals requires specimen preparation including staining the tissue to determine the location and concentration of the analyte. Usually, the invasive and destruction tests cannot be applied to record the dynamics of the distribution processes at a certain period. At present, the methods of the in vivo measurements undergo rapid development. Such approaches appear promising to make possible for researchers to measure in a point of the research object or to do imaging of the entire body and revealing the related biological processes in the living animals in dynamics. In most cases, it needs minimally invasive intervention or does not need one at all.[1] In this situation the optical diagnostics, and photonics-based techniques are of particular interest in the field of research because of their high sensitivity, versatility, and low cost. To obtain reliable information from an intact organism, various optical methods have already been developed and used for many years in the community of drug discovery to conduct studies in vivo.[2] For example, previously optical coherence tomography (OCT) was effectively used to assess the effectiveness of delivery of a percutaneous vaccine.[3,4] Intravital microscopy (IVM) was used for the non-invasive continuous

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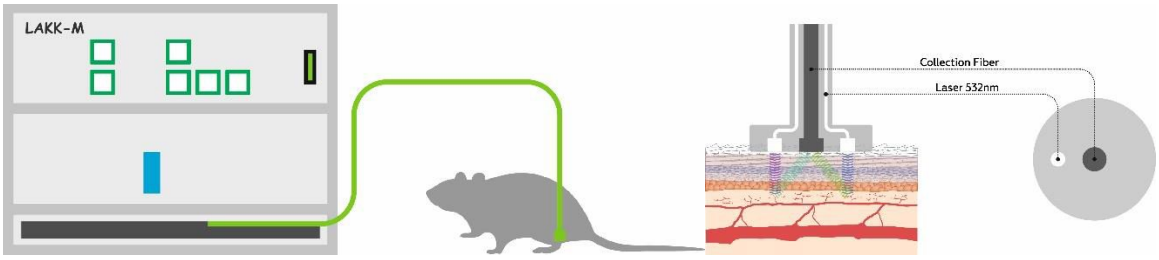
monitoring of drug movement in the body[5] as well as the photoacoustic (PA) imaging has been successfully applied to visualize the transport and accumulation of substances in the organ or tumour tissue.[6,7] The laser-induced fluorescence techniques also can be assigned to the group of methods. The diagnostic approach has considerable potential for drug discovery, not only during the development of a new drug formula but also in preclinical and clinical trials.[8,9] Optical methods, in particular, fluorescent spectroscopy, are promising tools for diagnostics in the modern medical practice as well[10].

Another vibrant area of modern study is targeted delivery of medicines, which can significantly improve the effectiveness of treatment of various diseases, reduce the occurrence of possible adverse side effects and disease complications. The creation of such controlled-release preparations in many cases is associated with the development of a microscopic nanoscale system, such as capsules for loading biologically active substances and delivering them directly to the target. Thus, encapsulation of biologically active materials for drug delivery in vivo is a complex task requiring significant fundamental and applied research including the development of nanocapsules for the substance transportation as well as the technology of in vivo control of the delivery in the body. One of the recent advancements in the field is the creation of porous inorganic nanoparticles that will have a high chemical and mechanical stability, being at the same time extremely flexible in their physical and chemical properties. As a transport container, the particles can be loaded with various components. Besides the labelling by a fluorescent agent makes it possible to control the delivery efficiency by optical method.[11,12]

In this study, using a fluorescence spectroscopy the distribution the circulation of the fluorescent labelled polymer particles in the blood circulatory system was assessed in a rat model. The study aimed to find informative points (areas) on the rat skin for transcutaneous fluorescence measurements and to investigate the dynamics of the propagation of the fluorescent-labelled (Rhodamine TRITC) nanocapsules injected in the circulatory system.

2. THE METHOD OF RESEARCH

The fluorescence spectroscopy system with fibre-optical probe "LAKK-M" (SPE "LAZMA" Ltd, Russia) was used for the in vivo measurements[13,14]. The system provides multiwavelength excitation, detects emission, and processes the fluorescence signal. Its light sources include fluorescence excitation in UV (wavelength = 365 nm, power = 1.5 mW), blue (wavelength = 450 nm, power = 3.5 mW) and green light (wavelength = 532 nm, power = 4.5 mW). The abovementioned fluorescence excitation powers are provided at the tip of fibre probe, which induces an excitation light flux in the tissue of no more than 0.16 W m^{-2} for 365 nm, 0.37 W m^{-2} for 450 nm and 0.47 W m^{-2} for 532 nm. The spectrometer was a polychromator with a diffraction grating, and a CCD line (TCD1304AP, Toshiba, Tokyo, Japan) was used as the detector.



a) RAT b)

Figure 1. Exterior view of the experimental setup (a) and the fibre diagnostic probe (b) used in the study

The microcapsules were prepared by using calcium carbonate ($CaCO_3$) particles as a sacrificial template. $CaCO_3$ particles were prepared according to the standard method described by Parakhonskiy et. al.[12] The $CaCl_2$ (0.33 M) and Na_2CO_3 (0.33 M) aqueous solutions, which was dissolved 20ml ethylene glycol, mixed under vigorous stirring for 3h, leading to the precipitation of $CaCO_3$ particles. When the process was finished, a $CaCO_3$ washed with pure water to remove the unreacted species. The spherical $CaCO_3$ particles with an average diameter of 500±100 nm were prepared. The structure of polymeric capsule shells included biodegradable polyelectrolytes dextran sulfate (DsS, MW > 70 000), poly-L-arginine hydrochloride (PARG, MW > 70 000). The combination of PARG and DS is mostly used for preparation bio-capsules via layer-by-layer method[15]. The process of layer-by-layer polymer film assembly is based on the interaction and self-organization of complementary macromolecule pairs with the formation of a water-insoluble complex on the template surface. The process begins with the adsorption of the polycation (pArg) from the aqueous solution onto the negatively charged surface of the

template. At the next step, the polyanion (DS) is adsorbed onto the positively charged surface of the template and again the sign of the surface charge of the template changes to negative. A step-by-step repetition of the described procedure leads to the formation of the template surface of a water-insoluble polyanion/polycation complex (Fig. 2a). After $CaCO_3$ core was removed by ethylenediaminetetraacetic acid disodium salt (EDTA). The capsules were labelled with a fluorescent dye rhodamine TRITC (Fig. 2b). Tetramethylrhodamine (TRITC) is a bright red-fluorescent dye with excitation ideally suited to the 557 nm laser line. Using this wavelength, we can observe all epidermis as well as a papillary layer of the dermis, so it allows validating the presence of the nanocapsules deep in microcirculatory vessels of the skin. The characterization of obtained microcapsules was carried out using confocal laser scanning microscopy (CLSM), scanning electron microscopy (SEM).

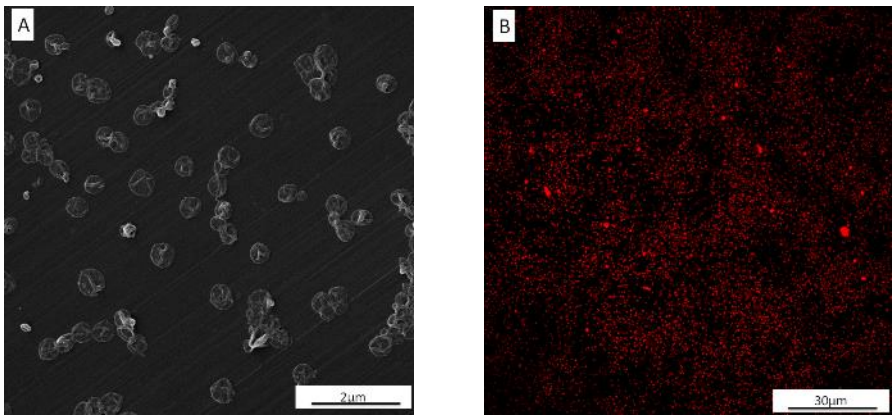


Figure 2. Capsules morphology analysis. Scanning electron microscopy (SEM) (a) and confocal laser scanning microscopy (CLSM) (b) of polyelectrolyte nanocomposite capsules. The SEM measurements (a) demonstrated the integrity of the nanofunctionalized shells and hollow inner cavity. The scan from CLSM analysis (b) was obtained using the spectrum excitation and emission for rhodamine TRITC dye. The CLSM data indicated low microcapsule aggregation and their spherical shapes and confirmation of high fluorescent signal

Experimental studies were carried out on groups of clinically healthy Wistar rats, formed by the analogue method. The animals were held in quarantine for two weeks in the vivarium of the Centre of Preclinical Research, CJCR "Retinoids", with temperature, humidity, bacterial contamination and day-night cycle control conditions under the ethical committee control concerning the Good Laboratory Practice Principles (GLP). Each day during the quarantine the animals were observed and supervised by the veterinary doctor, after that, they were randomised into two groups in according with their weight medians. During the experiment, the rats were anaesthetised with Zoletil 100 (Virbac, France) in standard doses. The model animals were divided into two groups, which outside analysis received the same food. In the study twelve 100-120 g, Wistar rats were divided into two groups: treated with rhodamine-loaded capsules, injected directly into the bloodstream (n = 6), and the control one (n = 6). Before the administration of the drug, a background fluorescence measurement procedure was performed at one excitation wavelength of 532 nm (Fig. 1b), and due to the better repeatability, the points of measurements on rats' thigh were selected. Treated group of rats received injections of rhodamine particles into the tail vein. The concentration of the resulting rhodamine in the group was 5 mg/kg of animal weight. Fluorescence spectra were recorded from thighs of anaesthetised rats during 90 min with 10 min intervals. Preliminary series of the measurements of the repeatability of the skin fluorescence intensity was conducted in control group. Before each measurement, the skin was previously depilated and cleaned with 96% ethanol solution. After an experiment, the animals were euthanised in the CO_2 cage.

The experimental studies complied with EU Directive 2010/63/EU, which defines a human attitude towards animals and refers to the Three Rs principles (replacement, reduction, and refinement). All studies were approved by the Ethical Committee of Orel State University.

3. RESULTS AND DISCUSSION

The obtained fluorescence spectra show a statistically significant increase in the fluorescence intensity in a group of rats received nanocapsules with rhodamine. In this group, a significant increase (from 42±5 to 100±7 a.u., twofold to the baseline level) at the wavelength of the peak of the rhodamine TRITC fluorescence intensity (about 590 nm) was registered (Fig. 3).

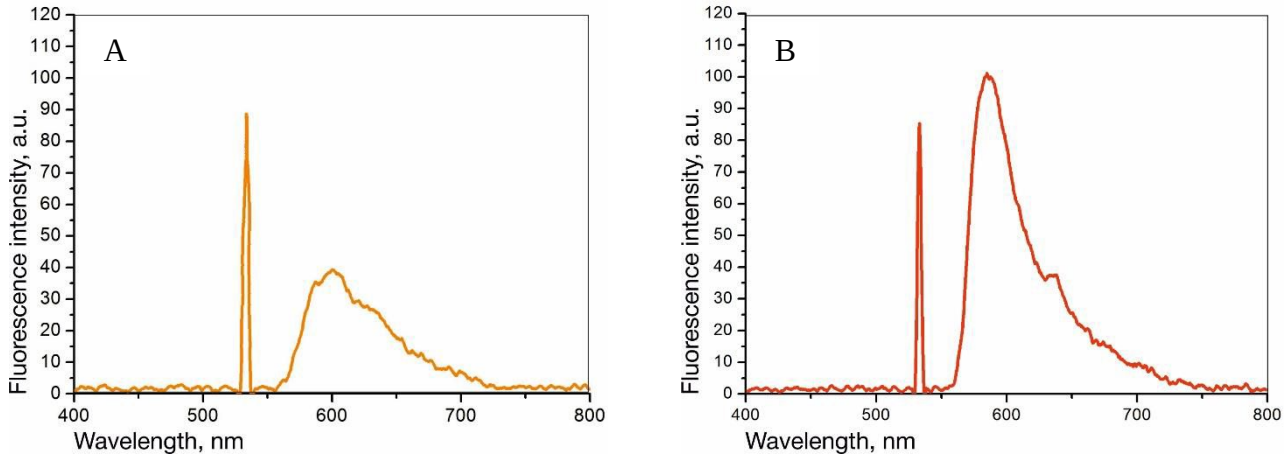


Figure 3. Fluorescence spectra in the control group (a) and in the group received fluorescent-labelled (rhodamine TRITC) nanocapsules (b)

The fluorescence intensity maximum were normalised by the:

$$k_f = \frac{I_{\max}}{I_{bs} + I_{\max}} , \quad (1)$$

Where k_f – normalised fluorescence intensity; $I_{\max}$ – represents registered fluorescence intensity at 590 nm; I_{bs} – represents the maximum intensity of the backscattered laser radiation (532 nm).

The normalised procedure is necessary to compensate variable absorption in the skin and to get more reliable measurement results (Fig. 4).

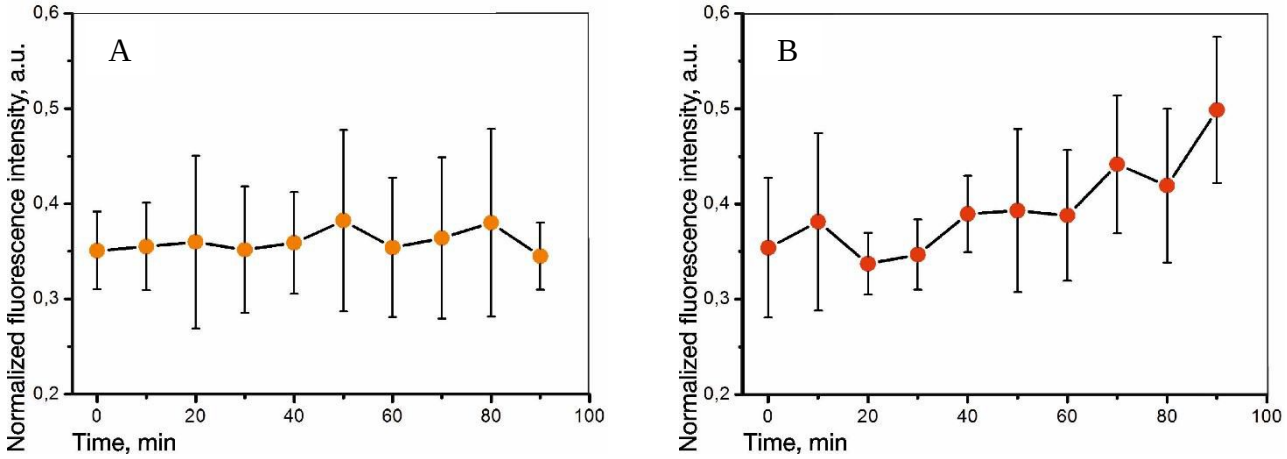


Figure 4. Dynamics of the intensity of normalized fluorescence in the control group (a) and in the group received fluorescent-labelled (rhodamine TRITC) nanocapsules (b)

Conducted processing of the registered experimental spectra has shown that the immediate transient process after particles administration is feebly marked. It can be explained by the relatively small impact of the direct dye fluorescence from the blood in the overall registered signal. The similar result by the same probe was obtained by the authors for liposomal particles in previous studies.[8,9] The diagnostic volume and depth for the fluorescence measurements for the different excitation wavelengths for the fiber optical probe were also assessed by the Monte Carlo modelling previously.[16]

Based on the results of the study, it was found that the nanocapsules contribute to the distribution and accumulation of the dye in tissue. Over time, the fluorescence of the tissue increases significantly in the group where the rats received

particles. Previously it was shown, that the size of the particles plays a crucial role in the ability to leave the blood stream.[17] The particles of the size below than 500 um can penetrate through the vessel wall (especially through the capillary wells).[18] Thus, the observed rising trend of the normalised fluorescence in the group received the particles can be explained by the accumulation of the dye in the skin layers. In case of the application of patch-based microneedles with rhodamine loaded biodegradable polymer particles in the rat model the 50% increase of the amount of permeated dye in the skin can be achieved after a lapse of 5 hours.[19] In the study, the 50% increase of the skin normalised fluorescence was achieved after 1.5 hours. In case of the liposomal fluorescein loaded particles, the same level of increase was observed by authors after 0.5 hours.[8]

4. CONCLUSION

In this study, we used the fluorescence spectroscopy to evaluate the penetration efficiency of nanocapsules from the circulatory system into adjacent tissues. Since porous containers have the potential to capture a significant amount of a fluorescent dye, one of methods for evaluating the effectiveness of transport can be measurements of the fluorescence intensity in the place of the body. The obtained fluorescence spectra show a statistically significant increase in fluorescence intensity in a group of rats that received nanocapsules with rhodamine. In this group, a significant increase (twofold of the baseline level) in the peak fluorescence intensity of used dye from 42±5 to 100±7 a.u. was registered.

The results show that the fluorescence spectroscopy can be used to transcutaneously measure the concentration dynamics of labelled particles in vivo. The approach can increase the statistical significance and reliability of preclinical trials and reduce the required number of animals providing valuable information about pharmacodynamics and the optimal dosage of the drug. The results can be used in the field of preclinical drug research to control and ensure the possible drug-in-place delivery as well as in the process of high-throughput screening during their trials. Future studies will be focused on the implementation of targeted delivery - the creation of directional transport systems for medicines delivered to the particular type of tissue.

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