Letter

Allocation of rhodamine-loaded nanocapsules from blood circulatory system to adjacent tissues assessed *in vivo* by fluorescence spectroscopy

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Abstract

Modern fluorescent modalities play an important role in the functional diagnostic of various physiological processes in living tissues. Utilizing the fluorescence spectroscopy approach we observe the circulation of fluorescent-labelled nanocapsules with rhodamine tetramethylrhodamine in a microcirculatory blood system. The measurements were conducted transcutaneously on the surface of healthy Wistar rat thighs *in vivo*. The administration of the preparation capsule suspension with a rhodamine concentration of 5 mg kg^{-1} of the animal weight resulted in a two-fold increase of fluorescence intensity relative to the baseline level. The dissemination of nanocapsules in the adjacent tissues via the circulatory system was observed and assessed quantitatively. The approach can be used for the transdermal assessment of rhodamine-loaded capsules *in vivo.*

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

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1. Introduction

The conventional monitoring of administrated substance distribution in animals requires specimen preparation including the staining of the tissue to determine the localization and concentration of various analytes. Typically, invasive and destructive tests cannot be applied to record the dynamics of the distribution processes within a certain time. Therefore, methods for whole animal imaging *in vivo* have undergone rapid development [1]. Such approaches appear promising, making it possible to measure a single point of the sample or to perform the imaging of the entire animal body and reveal the related biological processes in real time. In major cases, minimally invasive intervention is required [2]. From this point of view, optical noninvasive diagnostic approaches are of particular interest because of their high sensitivity, versatility and low cost. To obtain reliable information *in vivo*, various optical methods have been developed and successfully used in various medical applications in the past [3, 4]. Optical coherence tomography has been effectively utilized to assess the efficiency of percutaneous vaccine delivery [5, 6]. Intravital microscopy has been used for the noninvasive continuous monitoring of blood flow [7, 8] and drug transfer in the body [9]. Photoacoustic imaging has been successfully applied to visualize transport and the accumulation of substances in organs or in tumour tissue [3]. Laser-induced fluorescence techniques can also be assigned to monitoring methods inside the body utilizing microencapsulated biomarkers [10, 11]. The optical diagnostic approach also has considerable potential in preclinical and clinical trials [12].

Another vibrant area is the targeted delivery of drugs, which is able to significantly improve the effectiveness of the treatment of various diseases, and reduce the occurrence of possible adverse side effects and disease complications. The development of such controlled-release preparations, in many cases, is associated with the design of a microscopic nanoscale system, such as capsules for loading biologically active substances and delivering them directly to the target. Thus, the 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 drug transportation as well as *in vivo* delivery control technology [13, 14]. One of the recent advancements in the field is the fabrication of porous inorganic nanoparticles with high chemical and mechanical stability, as well as tunable physical and chemical properties. As transport containers, the particles can be loaded with various components. Fluorescent labelling makes it possible to optically monitor delivery efficiency [15, 16].

In the current study, fluorescence spectroscopy was used to assess the allocation of fluorescently labelled polymer nanoparticles in the blood circulatory system of healthy rats. The aim of the study was to find informative points (areas) on the rat skin for transcutaneous fluorescence measurements, and to study the dynamics of the fluorescent-labelled (rhodamine tetramethylrhodamine (TRITC)) nanocapsules injected into the circulatory system.

2. Materials and methods

The fluorescence spectroscopy system with a fibre-optical probe 'LAKK-M' (SPE 'LAZMA' Ltd, Russia) was used for the *in vivo* measurements (see figure 1). The system provides multiwavelength excitation, registers emission and processes the detected fluorescence signal. It includes fluorescence excitation in UV ($\lambda = 365$ nm, 1.5 mW), blue ($\lambda = 450$ nm, power = 3.5 mW) and green light ($\lambda = 532$ nm, power = 4.5 mW). The above-mentioned fluorescence excitation powers are provided at the tip of the fibre probe, which induces an excitation light flux in the tissue of no more than 0.16 W m^{-2} for 365 nm and 0.37 W m^{-2} for 450 nm. The spectrophotometer was a polychromator with a diffraction grating and a CCD (TCD1304AP, Toshiba, Tokyo, Japan) as a detector.

Polymeric capsules were fabricated using calcium carbonate ($CaCO_3$) particles as a sacrificial template. The $CaCO_3$ particles were prepared according to the standard method described by Parakhonskiy *et al* [15]. The $CaCl_2$ (0.33 M) and Na_2CO_3 (0.33 M) aqueous solutions, dissolved in 20 ml ethylene glycol, were mixed under vigorous stirring for 3 h, leading to the precipitation of $CaCO_3$ particles. Then, the $CaCO_3$ particles were washed with pure water to remove the unreacted species. Spherical $CaCO_3$ particles with an average diameter of 500 ± 100 nm were obtained. The structure of the polymeric capsule shells included biodegradable polyelectrolyte dextran sulfate (DS, MW $> 70\,000$) and poly-L-arginine hydrochloride (PARG, MW $> 70\,000$). A combination of PARG and DS is mostly used for the preparation of bio-capsules via the layer-by-layer method [11]. The process of layer-by-layer polymer film assembly is based on the interaction and self-organization of complementary macromolecular 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 becomes negative. A step-by-step repetition of the described procedure leads to the formation of a water-insoluble polyanion/polycation complex on the template surface (see figure 2(a)). Further, the $CaCO_3$ core is removed by ethylenediaminetetraacetic acid disodium salt. The capsules were labelled with fluorescent dye rhodamine TRITC (see figure 2(b)). TRITC is a bright red fluorescent dye with excitation ideally suited to the 532 nm laser line. Using this wavelength, we can observe the whole epidermis as well as a papillary layer of the dermis, which allows the presence of the nanocapsules in the microcirculatory system of the skin to be validated. Characterization of the obtained microcapsules was carried out using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) (see figure 2).

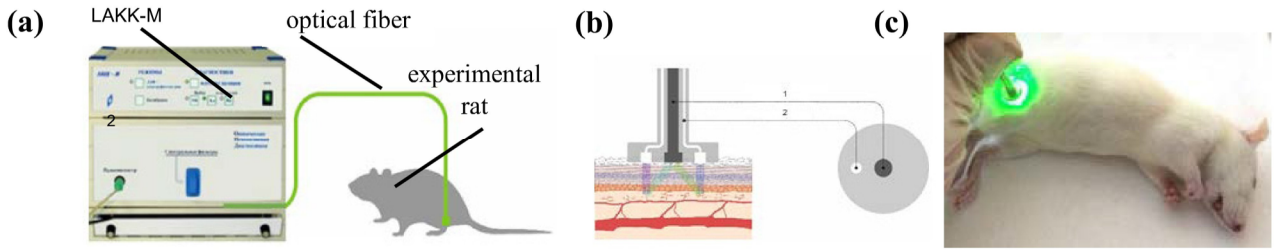

Figure 1. Exterior view of the experimental setup: (a) the multifunctional laser diagnostic complex LAKK-M control unit with laser and optical fibre; (b) the design of the fiber diagnostic probe used in the study ((1) is the collection fiber, (2) is a laser of 532 nm); (c) the laboratory rat used in the experiments.

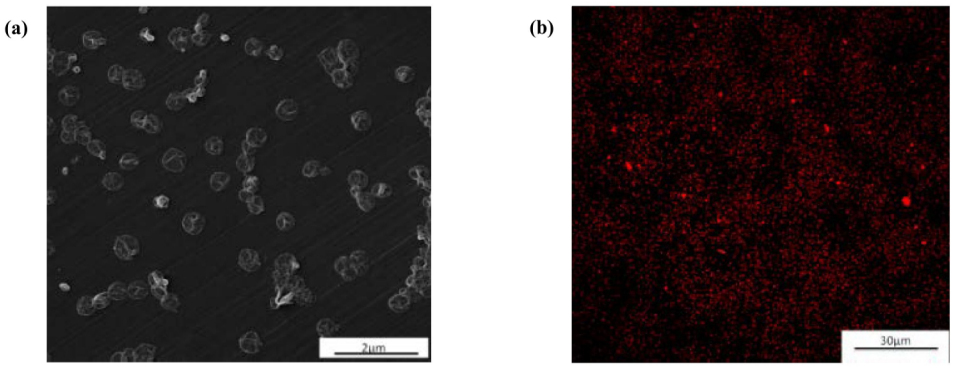

Figure 2. Capsule morphology analysis. SEM (a), and the CLSM (b) of the polyelectrolyte nanocomposite capsules. (a) The SEM measurements demonstrated the integrity of the nanofunctionalized shells and hollow inner cavity. (b) A CLSM scan at the emission bandwidth of the rhodamine TRITC dye.

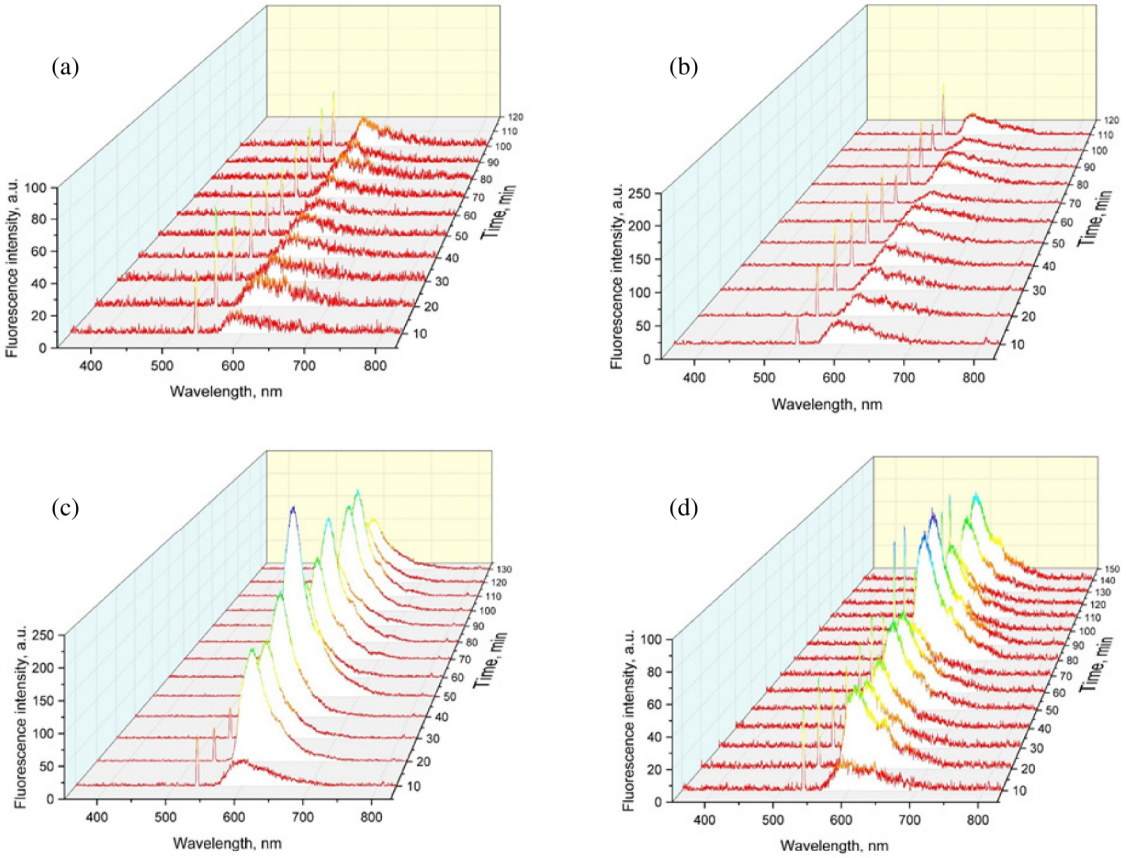

Figure 3. The averaged fluorescence spectra distribution of the leg ((a)—control rat group, (c)—capsule rat group) and tail ((b)—control rat group, (d)—capsule rat group).

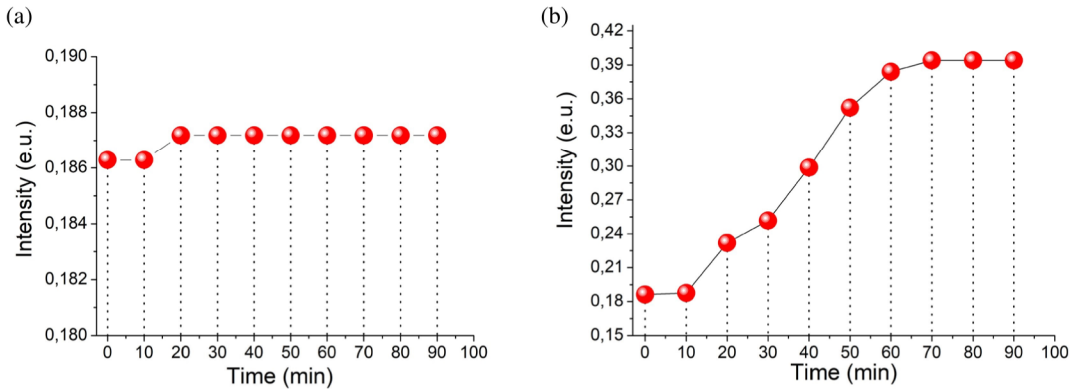

Figure 4. The dynamics of the intensity of normalized fluorescence in the control group (a) and in the group that received fluorescent-labelled (rhodamine TRITC) nanocapsules (b).

3. Results and discussion

Experimental studies were carried out on groups of clinically healthy Wistar rats. The animals were held in quarantine for two weeks in the vivarium of the Centre of Preclinical Research, JSC 'Retinoids', with temperature, humidity, bacterial contamination and day–night cycle conditions according to good laboratory practice principles. Every day during the quarantine the animals were examined by a veterinarian, after that they were randomised into two groups according to 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 received the same food. In the study, twelve 100–120 g Wistar rats were divided into two groups: one was treated with rhodamine-loaded capsules, injected directly into the bloodstream ($n = 6$), and the second was the control ($n = 6$). Before administration of the drug, background fluorescence was measured at a 532 nm excitation wavelength and due to the better repeatability, the measurement points on the rats' thighs were selected. The treated group of rats received injections of rhodamine capsules into the tail vein. The concentration of the resulting rhodamine in the group was 5 mg kg^{-1} of the animal weight. The fluorescence spectra were recorded from the thighs of anaesthetised rats for 90 min at 10 min intervals. A preliminary series of measurements of the repeatability of the skin fluorescence intensity was conducted in the control group. Before each measurement the skin was depilated and cleaned with 96% ethanol solution. After the experiment, the animals were euthanized in the CO_2 chamber. The experimental studies complied with EU Directive 2010/63/EU, which defines the human attitude towards animals and refers to the principles of the three Rs (replacement, reduction and refinement). All studies were approved by the Ethical Committee of Orel State University.

The obtained fluorescence spectra show a statistically significant increase in the fluorescence intensity in the group of rats that received nanocapsules with rhodamine. In this group, a significant increase (from 42 ± 5 to 100 ± 7 a.u., two-fold the baseline level) at the wavelength of the peak of the rhodamine TRITC fluorescence intensity (about 576 nm) was registered (figure 3)

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

where k_f is the normalised fluorescence intensity, I_{max} represents the registered fluorescence intensity at 590 nm, and I_{bs} represents the maximum intensity of the backscattered laser radiation from the tissue (532 nm). The normalised procedure is necessary to compensate the variable absorption in skin and get more reliable measurement results (figure 4).

4. Conclusion

In this study, we used fluorescence spectroscopy to evaluate the penetration efficiency of nanocapsules from the circulatory system into adjacent tissues. Since porous containers have the potential to accumulate a significant amount of fluorescent dye, one of the methods for evaluating the effectiveness of transport involves measuring the fluorescence intensity on the surface 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 (two-fold the baseline level) at the peak fluorescence intensity of the used dye ($\lambda = 590$ nm) from 42 ± 5 to 100 ± 7 a.u. was registered. The results show that 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 possible drug-in-place delivery as well as in the process of high-throughput screening during trials. Future studies will be focused on the implementation of targeted delivery—the creation of directional transport systems for medicines delivered to a particular type of tissue.

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