

# The development of skin immersion clearing method for increasing of laser exposure efficiency on subcutaneous objects

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## ABSTRACT

In this paper we have studied effect of a hyperosmotic optical clearing agent (OCA), such as polyethylene glycol, on the fluorescence intensity from a target located in subcutaneous area in the model experiments. As a fluorescence agent the nanocomposite including gold nanorods with hematoporphyrin was used. The remitted fluorescent signal traveling to the tissue surface was monitored over time as the tissue was treated with the OCA. The detected fluorescent signal increased as the scattering in tissue samples was substantially reduced. The study has shown how OCA can be used to improve the detected signal at localization of subcutaneous target tissue at the photothermal or photodynamic therapy. Immersion clearing of skin can be also useful for improvement of laser exposure efficiency due to the increasing of light penetration depth.

**Keywords:** fluorescence, visualization, gold nanorods, hematoporphyrin, skin, tissue optical clearing

## 1. INTRODUCTION

The using of a laser photothermal therapy (PTT), including selective photothermolysis, for tumor destruction [1], vessel coagulation [2], pathological bacteria suppression [3], and other therapeutic treatments is a new and perspective method. Suitably short pulses of selectively absorbed optical radiation can cause selective damage of pigmented structures, cells, and organelles *in vivo* [4]. The absorption of light by intrinsic chromophores, such as hemoglobin, melanin, and water, are typically used for the particular photothermal treatment. However, to enhance the effectiveness and selectivity of photothermal therapy as well as for photodynamic therapy (PDT) various exogenous dyes and nanoparticles are applied [1, 3, 5].

There are a plenty of clinically approved photosensitizers nowadays. For example, there are the indocyanine green activated by irradiation of an infrared diode laser [3,6], the methylene blue activated in red spectral range [7], the hematoporphyrin and chlorin derivatives [8], and many others photosensitizers. Carbon nanotubes are recognized as effective agents and used, for instance, for cancer cells labeling during the nanophotothermolysis with long laser pulses [1].

The unique optical properties of plasmon-resonant particles open new possibilities for applications in PPT. The gold nanoparticles have been used in PTT because their absorption coefficient near the plasmon resonance is four

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or six orders of magnitude greater than those of other organic labels and photosensitizers. Besides, gold nanoparticles are photostable and nontoxic, and they can be easily conjugated to antibodies or proteins [9].

Local nanoparticle storage inside malignant tissue or around makes it possible to use gold nanoparticles for tumor PTT. The combination of nanoparticles with the photosensitizers will allow providing PDT of tumors and localizing tumors hidden by surface layers of skin owing to fluorescence of the dyes.

However, for *in vivo* applying, significant absorption and scattering of visible light by tissues become an important limiting factor. Its limitations include the necessity to use visible light or NIR for activation which has a limited penetration depth [10]. The increasing of laser light intensity for compensation of losses of the energy, can lead to damages in tissue. The ability to reversibly alter the optical properties of normally turbid tissues offers the potential for improving of PTT and PDT effectiveness as well as the quality of target visualization and exact localization during the therapies. An increase in light penetration depth will allow photons to reach deep-lying target more effectively. For temporary and reversible optical clearing of a tissue, hyperosmotic chemical agents can be used.

Optical clearing technology is based on refractive index matching of scatterers (collagen, elastin fibers, cell components) and the ground material (interstitial fluid and/or cytoplasm) of a tissue [11]. At the recent time this technique is successfully applied in many areas connected with need of improvement of visualization quality of hypodermic objects, such as blood vessels [12], tattoos [13], tumors [14] etc. A plenty of potentially biocompatible clearing agents have been investigated for the purpose of finding-out of their suitability for use *in vivo*. Widespread used agents are: glycerol [15], propylene glycol [16], glucose [17], polyethylene glycol [18, 19] etc.

The goal of this work is the development of optical clearing method for improvement of visualization of a tumor hidden by a superficial layer of skin and improvement of laser exposure in modeling experiments with rats.

## 2. METHODS AND MATERIALS

### 2.1. Nanocomposite

For the experiments described in this work, the nanocomposite consisting of gold nanorods (NRs) core and hematoporphyrine (HP) doped silica shell was used. The nanocomposite was provided by Institute of Biochemistry and Physiology of Plants and Microorganisms, RF. First of all, gold NRs with the diameter of  $11 \pm 1$  nm and the length of  $44 \pm 7$  nm were synthesized. Then silica coated NRs were prepared. The shell thickness was  $20 \pm 3$  nm. Finally, mesoporous silica with included HP molecules was coated on the particle surface. The complete thickness of silicon oxide layer increased up to  $32 \pm 5$  nm. Common width and length of nanocomposite particles was  $75 \pm 6$  and  $108 \pm 12$  nm, respectively. The images of nanocomposite particles obtained with electron microscope Libra 120 (Carl Zeiss, Germany) are presented in fig.1. The NRs concentration was  $18 \times 10^{14}$  particles in one liter. The HP molar mass amounted to 590 g/mole, one liter of nanocomposite suspension contained 7 mg of HP or  $70 \times 10^{17}$  molecules. Thereby it was  $3.8 \times 10^3$  molecules per one gold nanoparticle. The concentration of basic suspension was taken as 1 and then sequentially diluted with distilled water up to achievement the following concentrations: 1/2 (3.5 mg/L), 1/4 (1.75 mg/L), 1/8 (0.88 mg/L), 1/16 (0.44 mg/L).

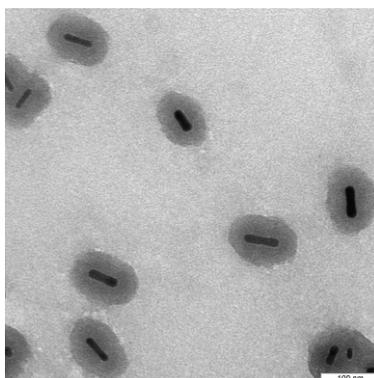


Fig. 1. TEM image of nanocomposites consisting GNR core and HP-doped silica shell.

The hypodermic injection of the nanocomposite suspension has modeled a labeled subcutaneous tumor because concentration of dye in a tumor at its intravenous introduction is higher, than in a surrounding tissue. Visualization was carried out by registration of a signal of fluorescence from nanocomposite.

## 2.2. Object of research

In experiments two white lab rats were used. Their hair was removed. The nanocomposite suspension was injected in the back area, the volume of suspension was 0.15 ml, the depth was about 1.5 mm. Subsequently animals were sacrificed and the skin samples with  $1.5 \times 1.5 \text{ cm}^2$  size were cut. The average thickness of samples was  $1.74 \pm 0.15 \text{ mm}$ . Study were performed *ex vivo*.

## 2.3. Optical clearing

Polyethylene glycol (PEG) with molecular mass 300 (Aldrich, USA) was used as an OCA. Samples were put in Petri dishes, filled up by OCA, and kept on thermo-table with constant temperature about  $42^\circ\text{C}$ . Meanwhile the OCA temperature inside the Petri dish was  $37^\circ\text{C}$ . Before each measurement samples have been taken out from Petri dishes and OCA was removed from the skin surface using filter paper.

## 2.4. Experimental setups

For measurement of fluorescence spectra of the HP solutions and the nanocomposite suspensions in a visible spectral range the spectrofluorimeter (Avantes, Netherlands) on the basis of the spectrometer Avaspec 2048 (200-1100 nm) and a source of light AvalightLED (7 mW, 405 nm) were used.

Fluorescence spectra of skin samples were measured by spectrometer USB4000 (Ocean Optics, USA). LED device (Polironik LTD, RF) was used as a light source (50 mW,  $400 \pm 10 \text{ nm}$ ). The angular aperture of an illuminating fiber was 0.03. Diameter of a fiber core was 1.5 mm.

At the tip of receiving fiber the collimator was fixed. For attaching of the both illuminating and receiving fibers, special holder was designed. The receiving fiber was fixed perpendicular to a skin surface and the illuminating one – at an angle  $20^\circ$  to it. Distance from the tip of the illuminating fiber to the skin surface was 2 mm, the exposed area was  $18 \text{ mm}^2$ . The area of irradiation collecting was  $12 \text{ mm}^2$ . The scheme of experimental setup is presented in fig. 2.

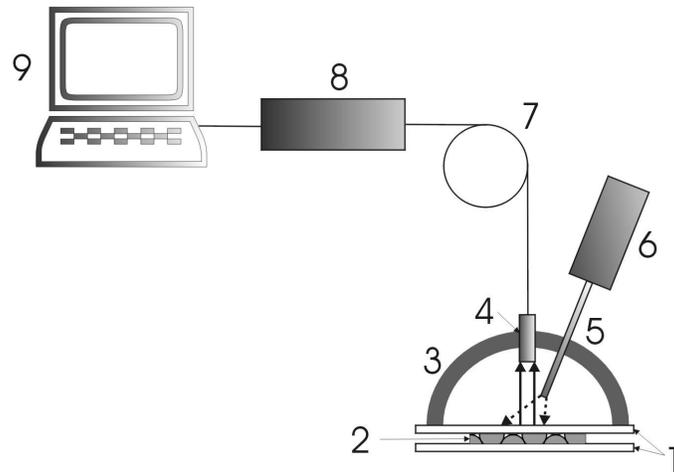


Fig.2. Experimental setup for the measurement of skin fluorescence spectrum: 1 is the object plates; 2 is the skin sample; 3 is the holder; 4 is the collimator; 5 is the illuminating fiber; 6 is the light source; 7 is the receiving fiber; 8 is the spectrometer; 9 is the PC.

The fluorescence spectra were measured before the samples putting in Petri dishes with the OCA, in 30 minutes, 1 hour and 2 hours after the beginning of the clearing process.

### 3. RESULTS AND DISCUSSION

Fluorescence spectra of nanocomposite (NR + HP) and HP solutions are presented in fig. 3. Results of measurements of fluorescence spectra of nanocomposite and HP solutions allow one to conclude: 1) the form of fluorescence spectrum differs for HP and nanocomposite solutions. Instead of two peaks at 615 and 675 nm, three peaks at 630, 650 and 690 nm appear and 2) the maximum magnitude of fluorescence for the solution is higher, than for the composite.

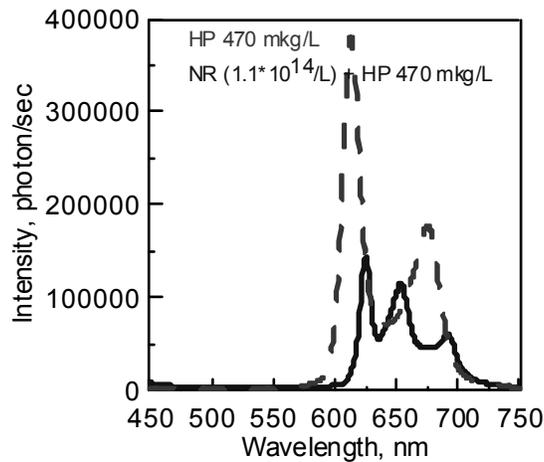


Fig. 3 Fluorescence spectra of nanocomposite (solid curve) on the basis of gold nanorods (NR) and HP in comparison with fluorescence of HP solutions (puncture curve) for the same concentration. For these measurements samples were dissolved in 16 times.

In Fig. 4 normalized fluorescence spectra for skin samples measured *ex vivo* after the injection of a nanocomposite suspension with 0.44 mg/L concentration before the optical clearing, in 30 min, 1 and 2 hrs after the beginning of the clearing are presented.

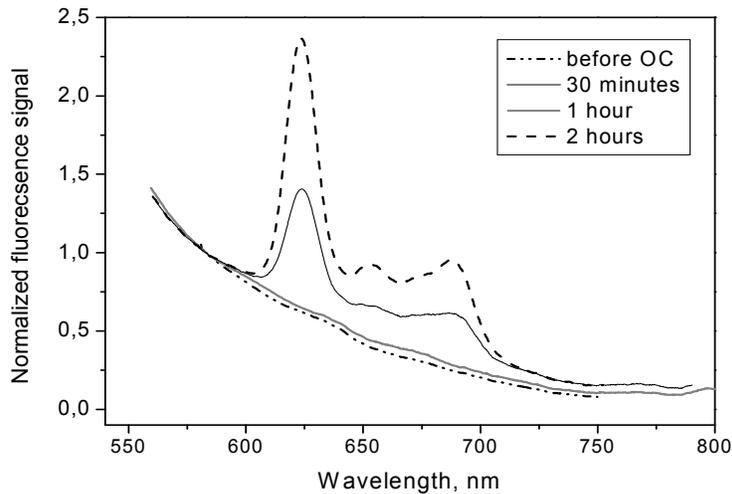


Fig. 4 Normalized fluorescence spectra of rat skin *ex vivo* after injection of nanocomposite (NR + HP) with concentration 0.44 mg/L in to living rat subcutaneous area.

It is well seen that at the first moment the presence of nanocomposite suspension in the skin didn't change the shape of the skin fluorescence spectrum. It could be explained by the fact that suspension was introduced into the skin at the depth about 1.5 mm, and the significant scattering of the superficial skin layers did not allow one to

visualize the suspension fluorescence. Under the OCA action the light scattering by superficial skin layers decreased and the shape of skin fluorescence spectrum in the range 600 – 750 nm changed in accordance with the nanocomposite fluorescence spectrum. The following peaks appeared: very low near 637 and 676 nm after 30 min, near 623, 655 and 688 after 1 hr and near 624, 653 and 687 nm after 2 hrs. In comparison with the spectrum of the nanocomposite presented in fig. 3, some shift of maxima of fluorescence peaks was observed. It can be explained by the interaction of HP with skin collagen.

The existence at 1-2 hrs of clearing of three peaks of hematoporphyrin corresponds to the spectra received by other researchers for this photosensitizer. However the magnitudes and wavelengths of peaks are different. For instance, in the study of HP derivative (HpD) binding to brain tumor cells *in vitro* (sterile stock solution of HpD (2.5 mg/mL) in PBS (pH 7.4)) HpD showed three fluorescence bands at 616, 636 and 678 nm [20].

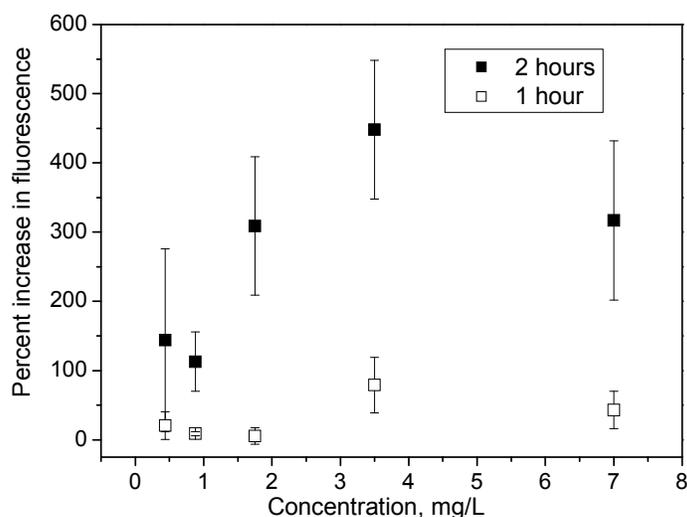


Fig. 5 Percent increase in fluorescent signal at the PEG action for five nanocomposite suspension concentrations: 7, 3.5, 1.75, 0.88 and 0.44 mg/L in an 1 hr (white squares) and in 2 hrs (black squares) of optical clearing.

Nanocomposite suspension with all used concentrations applied to skin resulted in an increase in detected fluorescence signal. The effect of different concentrations of the suspension on the fluorescent signal is shown in Fig. 5. It is well seen that magnitude of the percent increase in fluorescence of nanocomposite suspension with concentration of 7 mg/L is lower than that of nanocomposite with 3.5 mg/L concentration for both cases of 1 and 2 hrs of OCA application. It can be explained by the following most likely causes: differences in the depth of nanocomposite injection, differences in the skin thickness, the nanocomposite could be distributed in tissue in different ways, bleaching of HP during the measurements, or some others. It is also possible that some concentration suppression of fluorescence may take place. As we can see even the smallest nanocomposite concentration, 0.44 mg/L, gives, nevertheless, significant enhance in fluorescence signal after an hour (about 20%) and 2 hours (more than 140%) of optical clearing.

The largest percentage of increase in fluorescence was observed at the nanocomposite suspension concentration 3.5 mg/L: ~80% in an hour and ~450% in 2 hrs of OCA action. Refractive index matching effectively damps light scattering, and thus absorption of light is also reduced due to the lower number of photons circulating within a tissue. Significant dispersion of the obtained results caused apparently by differences in depths of injection and small quantity of samples, just two animals were investigated.

The received results support the general positive dynamics in research of impact of clearing agents on fluorescence signal intensity. For instance, in experiment with the hamster dorsal skin where fluorescence measurements were performed with OCA applied to the subdermal side of the skin and rhodamine fluorescent film placed against the same skin side, on average, up to 100% increase in fluorescence intensity was seen for 20-min glucose and glycerol applications, and up to 250% for DMSO [21]. Detection of *Salmonella typhimurium* fluorescence through porcine skin gave up to 26-time emission increase through a 3-mm mature gilt porcine skin to fourth hour of 100% glycerol topical treatment [22]. A considerable improvement of fluorescent and Raman signals was recently demonstrated for *in vivo* animal models [22, 23]. Computer simulations presented in Ref. [24] have also shown that the effect of refractive index matching at the skin interface produces a remarkable enhancement of detected fluorescence volume localization.

In the experiment with fresh porcine skin after the applying of 50% glycerol for 30 min the detected intensity of chemiluminescence from treated skin tissue was approximately fivefold stronger than that from untreated skin [25]. The results of the *in vivo* simulated bioluminescence experiment were a 41.8% decrease in integrated intensity and a 38.5% decrease in spot size that were seen 30 min postinjection of glycerol, that was also associated with reduction of scattering coefficient and OCA action [26]. Reduction of the integrated intensity detected by a distant CCD camera was explained by reduction of multiplicity of scattering which may decrease luminescence outcome if a light source is inside scattering medium, as it was recently shown directly for autofluorescence signals in optically cleared tissues [27].

According to the empirical results, referred above, optical clearing of skin *ex vivo* can promote exact localization of the studied area stained by a nanocomposite suspension, even at its minimum concentration in skin on depth more than 1 mm. In some cases fluorescence of suspension was registered in 30 min after the beginning of OCA action. The significant optical clearing effects were observed at the expense of OCA immersion needed and of thickness reduction of samples at tissue dehydration during the clearing process.

## 5. CONCLUSION

The results of this study showed that the optical skin clearing is a robust technology for increasing of the light penetration pathlength within a tissue. Consequently we have an opportunity to localize tumor more correctly if it is marked by a fluorescent dye or photosensitizer. This effect could be also used for more efficient laser irradiation targets hid under the skin surface at photothermal or photodynamic therapy.

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