In vitro study of Indocyanine Green solution interaction with skin

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ABSTRACT

Absorption properties of Indocyanine Green (ICG) dissolved in both water and 40%-ethanol for potential using in laser selective thermolysis have been researched. Correlation between magnitudes and positions of absorption bands of ICG in dependence on the kind of solvent and concentration of ICG has been studied. Interaction between ICG solutions and rat skin *in vitro* has been investigated. Shift of the main absorption peaks of ICG solutions in skin has been defined. Such shift is caused by ICG interaction with protein molecules of the stratum corneum and dermis. Diffusion coefficients of ICG in skin have been estimated at diffusion of aqueous solutions through dermis and at diffusion of alcohol solutions through both dermis and epidermis as $(7.70\pm2.51)\times10^{-7}$, $(18.79\pm3.35)\times10^{-7}$ and $(6.85\pm3.75)\times10^{-7}$, respectively.

Keywords: Indocyanine Green, skin, absorption coefficients, absorbance spectra, diffusion coefficients

1. INTRODUCTION

Control of absorption properties of living tissues is a method widely used in diagnostics as well as therapy and surgery. Effective change of absorption properties of living tissues can be provided by biocompatible dyes. It is well known the application of biocompatible fluorescent dyes as contrast agents for imaging of blood flows,[1,2] visualisation of atherosclerotic plaques[3] and localisation of hidden tumours.[4] Photodynamic therapy utilizes photosensitive dyes and light. It is known successful use of photodynamic therapy for the treatment of both cancer[5-8] and infectious diseases such as acute and chronic maxillary sinusitis,[9,10] paradontitis[11] and gastric infection.[12] Dyes can be applicable for local increase of tissue absorption that is used for precise microsurgery[13] selective laser thermolysis of tumours,[14,15] treatment of skin and follicle lesions,[16-20] etc.

Indocyanine Green (ICG) as a biocompatible dye is widely used in various areas of medicine. ICG has chemical formula $C_{43}H_{47}N_2O_6S_2Na_2$ and molecular weight 775. It is tricarbocyanine type of dye with absorbing properties in red – infrared spectral range.[1,21] It has two absorption bands. One of them can place in the visible or in the NIR range and another one places in the NIR range. The long-wavelength band is corresponded to monomer form of the dye and short-wavelength one is corresponded to dimer form. In aqueous dye solutions dimerization is observed at concentration 10^{-7} mol/cm^3.[22] It is used as a diagnostic aid for blood-volume determinations as well as cardiac- and liver-function test,[1,2,21,23,24] retinal and choroidal angiography,[25,26] tumor imaging,[4] burn depth estimation[27] and other. ICG is widely used as the photosensitizer in photodynamic therapy[28-30] and as absorber of laser energy in photothermal therapy[14,15,31-33] of tumors as well as in laser welding[34-37] and repair of tissues[38,39] because of its high absorption at wavelength of the powerful laser diodes. The dye can be successfully used in cosmetology, for example, for acne treatment and hair removal.[17-20]

The principal advantages causing the acceptance of this dye in medical practice are the presence of the absorption maximum near the isobestic point of hemoglobin and oxyhemoglobin around λ = 800 nm, the confinement to the vascular compartment by binding to plasma proteins, the very low toxicity, and the rapid excretion, almost exclusively into the bile.[23] ICG has somewhat bizarre light absorption behavior as a function of concentration because of it tends to aggregate in water at high concentrations.[21,23] This means that the effective absorption does not increase linearly with increase of concentration. Furthermore, magnitudes and positions of absorption bands of ICG depend on the solvents.[21,40]

ICG can interact with components of biological tissues and blood. One of the manifestations of such interaction is a shift of the absorption band maximum of the dye. It was established that binding of the dye molecules to various organic molecules in biological tissues leads to a shift of the absorption peak toward longer wavelengths. The absorption peak

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maximum was observed at 805 nm in the human blood[26] and human skin *in vivo*[41] and at 810 nm in the epidermal cell cultures and epidermal strips *in vivo*.[28,40,42] However, changes in the absorption spectra of ICG caused by its interaction with biological tissues upon their staining are not studied in details. We have performed this study to estimate shifts of the absorption bands of ICG dissolved in different solvents at the interaction of the solutions with skin. This study can be important to choice optimal wavelength of the laser irradiation.

2. METHODS AND MATERIALS

2.1. Materials

Skin samples have been taken from white rat in an hour *post mortem*. Both hair and subcutaneous fat layer have been removed. The area of the samples was about 3×3 cm^2. The thickness of each sample has been measured at initial moment with a micrometer in ten points and averaged. The average value was 0.87 ± 0.04 mm.

In this investigation we used Indocyanine Green (Aldrich Chemical Co., USA) and the follow solvents: distilled water and 40%-ethanol solution (expressed in the volume fractions).

2.2. Experimental setup

Spectra of the total transmission of all solutions in the 400-1000 nm wavelength range were recorded by a commercially available spectrophotometer CARY-2415 (Varian, Australia) with integration sphere. Transmission spectra of pure solvents were used as references. The thickness of used cuvette was 100 μm.

The measurements of rat skin reflectance have been performed in the spectral range 450-1000 nm using a commercially available optical multichannel spectrometer LESA-5 (BioSpec, Russia) with fiber-optical probe at room temperature about 20°C. The scheme of the experimental setup is shown in Fig. 1.

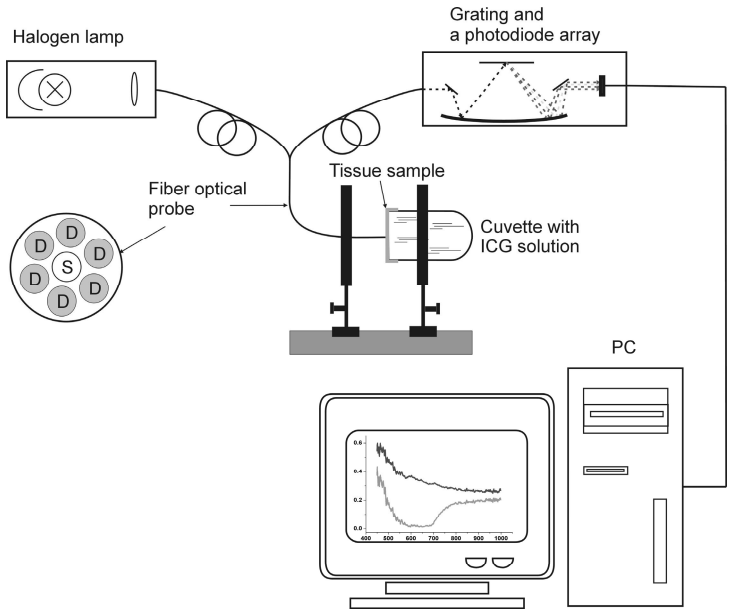

Fig. 1. Experimental setup for measurements of skin reflectance spectra. S and D mean source and detector fibers, respectively.

The fiber-optical probe consisted of the seven optical fibers. All fibers had 200 μm core diameter and a numerical aperture of 0.22. The central fiber (S) delivered incident light to the tissue surface and the six fibers (D) placed around the central fiber collected reflected light. Distance between the delivering and receiving fibers was 290 μm. As a reference a white slab $BaSO_4$ with a smooth surface was used. For the spectrophotometric measurements each sample was fixed on the special cuvette with solution of ICG. Penetration of the dye solution into skin was provided through

epidermis or dermis that modeled real conditions of photodynamic procedure. Measured reflectance spectra was recalculated in absorbance spectra with the relation:

$$A(\lambda) = -\ln(R(\lambda)),$$

where $A(\lambda)$ is the skin absorbance, $R(\lambda)$ experimental values of the time-dependent reflectance, λ is wavelength, nm.

2.3 Method for estimation of diffusion coefficient

Method for estimation of ICG diffusion coefficient is based on the time-dependent measurement of the tissue absorbance in the spectral range from 600 to 900 nm, which correspond to absorption bands of the dye. The transport of ICG within skin is described in the framework of free diffusion model. We assume that the following approximations are valid for the transport process:

1. only concentration diffusion takes place; i.e., the flux of the dye into the tissue, at a certain point within the tissue sample, is proportional to the ICG concentration at this point;
2. the diffusion coefficient is constant over the entire sample volume.

Geometrically the tissue sample is presented as a plane-parallel slab with a finite thickness. Since lateral sides of the experimental samples were fixed, the one-dimensional diffusion problem has been solved. Diffusion equation of the dye transport has the form:

$$\frac{\partial C(x,t)}{\partial t} = D\frac{\partial^2 C(x,t)}{\partial x^2}, \tag{1}$$

where $C(x,t)$ is the ICG concentration, g/ml; D is the ICG diffusion coefficient, cm^2/s; t is time, s; and x is the spatial coordinate, cm.

We also suppose that penetration of ICG into the tissue does not change the dye concentration in the external volume. Besides, due to geometry of the measurements, penetration of ICG into the skin sample takes place from top surface of the sample only. The corresponding boundary conditions are

$$C(0,t) = C_0 \quad \text{and} \quad \frac{\partial C(l,t)}{\partial x} = 0, \tag{2}$$

where C_0 is ICG concentration in external solution, g/ml, and l is skin sample thickness, cm.

The initial condition corresponds to the absence of ICG inside skin before the measurements,

$$C(x,0) = 0 \tag{3}$$

for all inner points of the sample.

Solution of Eq. 1 for a slab with a thickness l at the moment t with boundary (Eq. 2) end initial (Eq. 3) conditions has the form

$$C(t) = C_0\left(1 - \frac{8}{\pi^2}\sum_{i=0}^{\infty}\frac{1}{(2i+1)^2}\exp\left(-(2i+1)^2\, t\,\frac{\pi^2}{4}D\Big/l^2\right)\right), \tag{4}$$

where $C(t)$ is the volume-averaged concentration of ICG within skin sample.

In a first-order approximation Eq. 4 is reduced to the form

$$C(t) \approx C_0\left(1 - \exp\left(-t\pi D/l^2\right)\right). \tag{5}$$

For determination of ICG diffusion coefficient in skin the approach suggested in papers[41,43] has been used. The method is based on the use of modified Lambert-Beer law and in this case, tissue absorbance can be determined as

$$A = \mu_a\sigma\rho + G, \tag{6}$$

where μ_a is absorption coefficient, ρ is source-detector distance, σ is differential factor of photon path-length, taking into account the lengthening of photon trajectories due to multiple scattering, and G is constant, defined by geometry of the experiment. To simplify calculations, $\rho\sigma$ can be replaced by parameter L which is defined by both absorption and

scattering tissue properties, and source-detector distance. Since in this study the distance (290 μm) is commensurable with photon free path-length, parameter L is defined by tissue scattering properties only.[44-46]

Penetration of ICG into tissue increases the tissue absorbance in spectral range corresponding to absorption bands of the dye. Thus, the tissue absorbance measured in different time intervals can be determined as

$$A(t,\lambda) = A(t=0,\lambda) + \Delta\mu_a(t,\lambda)L\,, \tag{7}$$

where t is the time interval, λ is the wavelength, $\Delta\mu_a(t,\lambda) = \varepsilon(\lambda)C(t)$ is the absorption coefficient of ICG within tissue, $\varepsilon(\lambda)$ is ICG molar absorption coefficient, $C(t)$ is ICG concentration in tissue, and $A(t=0,\lambda)$ is the tissue absorbance, measured in the initial moment.

Thus, the equation

$$\Delta A(t,\lambda) = A(t,\lambda) - A(t=0,\lambda) = \Delta\mu_a(t,\lambda)L = \varepsilon(\lambda)C_0\left(1-\exp\left(-t\pi D/l^2\right)\right)L \tag{8}$$

can be used for calculation of the ICG diffusion coefficient.

This set of equation represents the direct problem, i.e., describes the temporal evaluation of the absorbance of skin sample dependent on ICG concentration within the tissue. Based on measurement of the evolution of the tissue absorbance, the reconstruction of the ICG diffusion coefficient in skin has been carried out. The inverse problem solution has been obtained by minimization of the target function as

$$F(D) = \sum_{i=1}^{N_t}\left(A(D,t_i) - A^*(t_i)\right)^2\,, \tag{9}$$

where $A(D,t)$ and $A^*(t)$ are the calculated and experimental values of the time-dependent absorbance, respectively, and N_t is the number of time points obtained at registration of the temporal dynamics of the absorbance. To minimize the target function the Levenberg-Marquardt nonlinear least-squares-fitting algorithm described in detail by Press et al.[47] has been used. Iteration procedure is repeated until experimental and calculated data are matched.

3. RESULTS AND DISCUSSION

Fig. 2 shows absorption spectra of ICG aqueous solutions with concentration 1 mg/ml and 0.1 mg/ml. It is well seen the maximum of ICG absorption is in shorter-wavelength region. It is mean that in aqueous solutions ICG is in dimer form. NIR absorption peak is observed at 769 nm, and it is significantly below then visible one. The peak position is not depended on the concentration of ICG in solution.

Fig. 3 shows absorption spectra of ICG dissolved in both pure ethanol and 50% ethanol with concentration 1 mg/ml, and in 10% ethanol with concentration 0.1 mg/ml. All peaks are shifted to NIR range in comparison with the spectra of aqueous solutions. It is well seen that magnitude and relative position of two absorption bands of ICG solutions are differed in dependence on concentration of the dye in the solutions. At the concentration 1 mg/ml maximum of absorption band is observed at 787 nm (monomer form). At that absorption coefficient of ICG solution in 50% ethanol is significant higher then the same in pure ethanol at equal concentrations. Another peak is less marked. It is observed at 720 nm. At the concentration 0.1 mg/ml both peaks are shifted to shorter-wavelength range and became comparable ones. Dimer dye form is prevalent due to water influence.

Besides, significant difference between the absorption coefficient values of aqueous solution of ICG and that of alcoholic solutions of ICG is observed. At the equal concentration of the dye in the both aqueous and alcohol solutions ICG-alcohol solution have higher absorption coefficient (437 cm^{-1}) at the wavelength 787 nm then aqueous solution (252 cm^{-1}) at 697 nm.

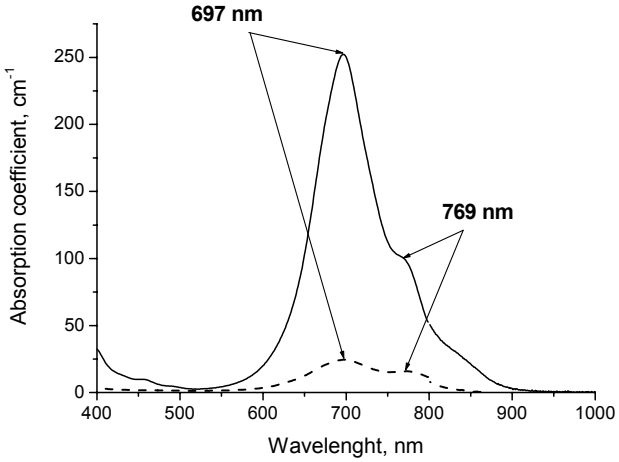

Fig. 2. Absorption spectra of aqueous solutions of ICG with concentrations 1 mg/ml – solid line and 0.1 mg/ml - dot line.

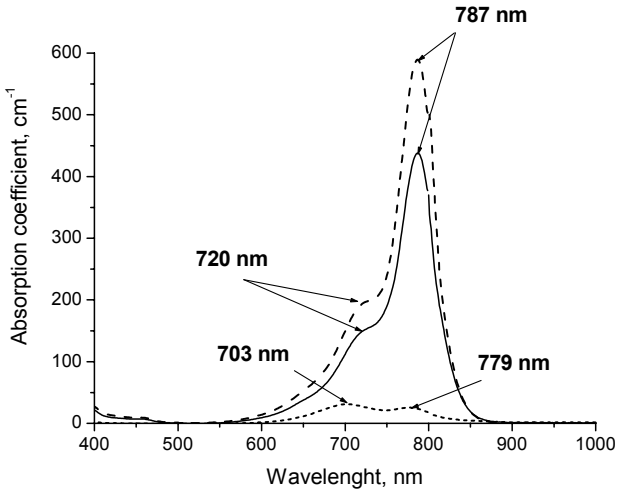

Fig. 3. Absorption spectra of ICG dissolved in pure ethanol with concentration 1 mg/ml – solid line, in 50%-ethanol solution with concentration 1 mg/ml - dot line, and in 10%-ethanol solution with concentration 0.1 mg/ml - dash line.

Figures 4 and 5 show absorbance spectra of skin measured in different moments when aqueous ICG solution contacted with dermis and epidermis, respectively. Diffusion of the dye through dermis has being gone on in about 330 minutes. Absorbance of the skin sample was increased during ICG penetration into the tissue. In the figure it is also seen, that shape of the spectrum is different for different moments. At the initial moment monomer is more visible. However during the dye diffusion the prevalence of dimer absorption band is observed. It is connected with increasing of ICG concentration inside tissue.

In Fig. 4 both spectra skin and dye solution are presented. From the figure it is well seen that absorption bands of ICG in skin are shifted related the absorption bands of ICG aqueous solution. The main maximum was shifted on 17 nm to shorter wavelengths. The second peak was observed at $\approx$790 nm and it was feebly marked. Its shift to longer wavelengths was about 21 nm. During the penetration of the ICG solution into the tissue the interaction between dye molecules and tissue components are taking place. As a sequence transformation of ICG spectrum is happened.

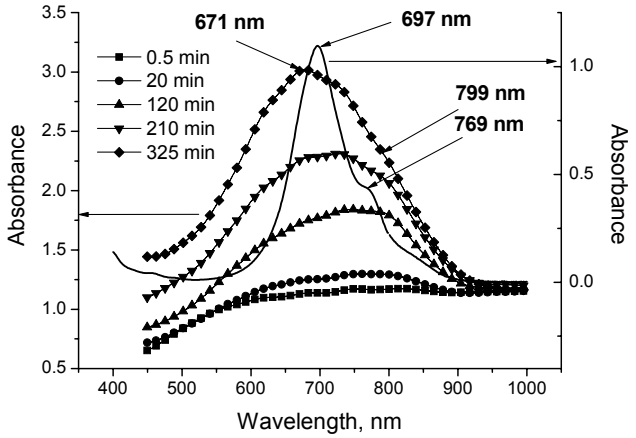

Fig. 4. Absorbance spectra of skin dyed by aqueous ICG solution. Diffusion of the dye was from dermis.

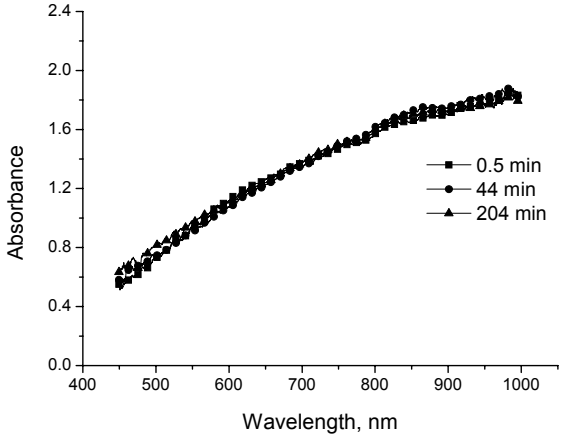

Fig. 5. Absorbance spectra of skin dyed by aqueous ICG solution. Diffusion of the dye was from epidermis.

Fig. 5 demonstrates dynamics of skin absorbance spectra at the ICG diffusion from epidermis. It is well seen that during the observation the skin spectra has not being changed under action of ICG solution. It is connected with low diffusion rate of ICG aqueous solution in epidermis in comparison with that in dermis as lipid barrier and structure of stratum corneum containing packed closely cell layers prevent to the diffusion. ICG is hydrophilic substance; therefore diffusion of its aqueous solution through hydrophobic lipid barrier of epidermis is difficult. For the enhancement of the dye penetration through lipid barrier 40% aqueous ethanol solution was used as a solvent.

In Figs. 6 and 7 absorbance spectra of skin dyed by 40%-ethanol ICG solution are presented. Maxima of skin absorption bands at the diffusion of the dye through dermis as well as through epidermis are observed at 806 and 690 nm. The peaks have not coincided with the absorption peaks of ICG in ethanol solution due to the interaction of the dye with tissue. The main maximum (806 nm) has shifted to longer wavelengths on 19 nm and the maximum at 693 nm has shifted to shorter wavelengths on 30 nm.

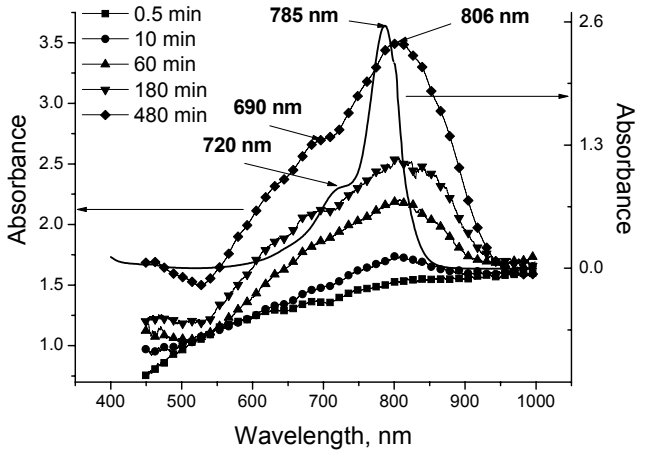

Fig. 6. Absorbance spectra of skin dyed by 40%-ethanol ICG solution. Diffusion of the dye was from dermis

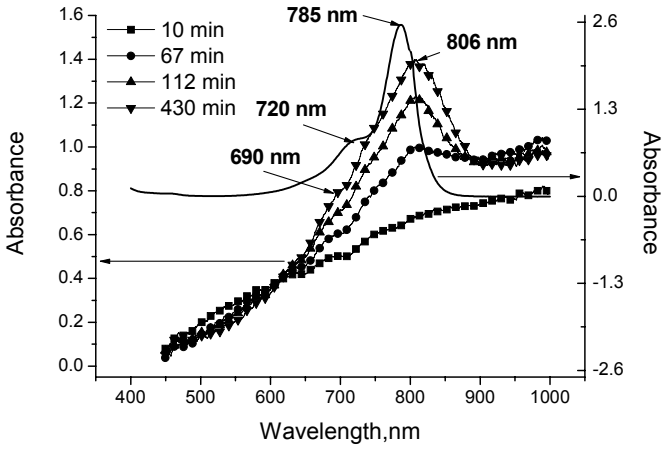

Fig. 7. Absorbance spectra of skin dyed by 40%-ethanol ICG solution. Diffusion of the dye was from epidermis

At the dye diffusion through dermis unlike one through epidermis absorption band of ICG placed in near UV range has significant effect on the form of the spectra. In Fig. 2 and 3 wing of UV absorption band is indicated as not great lifting of spectral curves. In Fig. 6 the wind is clearly shown in the range shorter then 520 nm. Fig. 4 also demonstrates appearance of the band at the increase of the dye concentration in skin tissue. At the dye diffusion through epidermis the band has not become apparent.

From the comparison of Figs. 6 and 7 it is seen that the absorption values and peak relation are differed at the diffusion of the solution through dermis or epidermis. It is connected, apparently, with different concentration of ICG in the samples. During approximately equal time interval the concentration of ICG in the first sample became significantly higher then in the second one.

From experimental data diffusion coefficients were estimated with the help of equations indicated above. The values are presented in the Table.

Table 1. Diffusion coefficients of ICG solutions in skin.

Solution	Thickness of the sample, mm	Diffusion coefficient, cm^2/c	
		diffusion goes on through dermis	diffusion goes on through epidermis
Aqueous ICG solution	0.93±0.07	$(7.70\pm2.51)\times10^{-7}$	–
40% ethanol ICG solution	0.80±0.01	$(18.79\pm3.35)\times10^{-7}$	$(6.85\pm3.75)\times10^{-7}$

Analysis of obtained results shows that ethanol addition in aqueous solution of ICG increases value of its diffusion coefficient through dermis more then 2 folds. The diffusion enhancement is connected with damage of lipid bilayer and increasing pore size of human epidermal membrane under ethanol action.[48] Therefore we could estimate diffusion coefficient of ICG at the interaction of the dye solution with epidermis only for ICG-ethanol solution.

4. CONCLUSION

In this study we have investigated absorption properties of Indocyanine Green dissolved in both water and 40%-ethanol for potential using in laser selective thermolysis. Values of absorption coefficient of the solutions have been obtained. The magnitude and the position of two absorption bands of ICG-solutions are differed in dependence on the kind of the solvent.

At the penetration of the ICG solution into the tissue the interaction between dye molecules and tissue components are taking place. As a sequence transformation of ICG spectrum in skin is happened. Besides dimerisation of ICG at the increasing of the dye concentration in tissue influences on magnitude and relation position of the main absorption bands. The shifts absorption peaks of ICG from the value obtained in the solutions, to both longer and shorter wavelengths due to ICG binding with proteins in the human stratum corneum and dermis have been found.

Low diffusion rate of ICG aqueous solution in epidermis has not allow estimating diffusion coefficient in stratum corneum. Ethanol addition in aqueous solution of ICG enhances diffusion rate of the solutions. Diffusion coefficients of ICG were estimated at diffusion of aqueous solutions through dermis and at diffusion of alcohol solutions through both dermis and epidermis as $(7.70\pm2.51)\times10^{-7}$, $(18.79\pm3.35)\times10^{-7}$ and $(6.85\pm3.75)\times10^{-7}$, respectively.

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