In vitro and *in vivo* study of dye diffusion into the human skin and hair follicles

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ABSTRACT

We present experimental results on *in vitro* and *in vivo* investigation of dye diffusion into the human skin and hair follicles. It was shown that dyeing as a method of enhancement of the absorption coefficient of hair follicle tissue components can be used for selective photodestruction of hair follicle and surrounding tissues. Strength and depth of hair follicle dyeing inside the skin were determined for various dyes.

Keywords: human skin; hair follicles; diffusion; dyes; ICG.

1. INTRODUCTION

To remove unwanted or excess hair, the principles of selective photothermolysis[1] have been employed with several different laser and light instruments that permit the effective treatment of large areas of hair-bearing skin with minimal discomfort and with low risk of scarring or other complications.[2-11]

Dyeing, like a method of increasing of the absorption coefficient of biological tissue, can be used in photothermolysis. Selective absorption of laser radiance by dye induces intense heat and damage of cells.[6-11]

We have studied the efficiency of the human skin, hair shaft, and hair follicle dyeing. The dyeing solution should satisfy to the following requirements: 1) has a narrow absorbing band with a mean wavelength corresponding to the used laser wavelength; 2) has the absorption coefficient larger then that of ambient tissue; 3) being nontoxic and has nontoxic products of decomposition; 3) penetrates deeply into follicle; 4) being easy to delete from the skin surface. We have studied some dyes and solvents to choose the best fit to these requirements. Commercially available hair colorants, food dyes, and medical dyes have been studied. Absorption spectra of all dyes, dyed hair shafts, and skin were measured. Pre- and post-dyed epilated hair samples and biopsy hair samples were collected and analyzed.

2. HAIR STRUCTURE

The structure of hair and its changing in different stages are of great importance for in depth dyeing of hair shaft and follicle. We have studied the human body hairs from forearm and lower leg in various stages: anagen, catagen, and telogen. The hair in the anagen stage[12,13] continues to produce a hair shaft for a few months. During this period its structure remains constant. Structure of a fully developed anagen hair is shown schematically in Fig. 1. The expanded, bulbous section of the lower hair follicle, including the hair matrix and the dermal papilla, is known as the hair bulb. The isthmus of the follicle is the portion lying in between the point of attachment of the arrector pili muscle and the point of entry of the sebaceous gland duct. The infundibulum lies above the entry of the sebaceous duct and merges with the surrounding surface epidermis. Hair follicle length in different stages changes with development however area of follicle from arrector pili muscle to skin surface remains permanent.

Just external to the hair shaft are the three layers forming the inner root sheath (IRS). External to IRS is the clear-celled outer root sheath (ORS). In the lower portion of the follicle (below the isthmus), the ORS does not keratinized. The nonkeratinized inner layer of cells lies against the fully keratinized IRS. At the level of isthmus, where the IRS disintegrates, the ORS keratinizes.[12,13] Hair follicle length in anagen stage is about 3 mm. The hair canal, infundibulum, and the sebaceous gland are a path for in depth penetration of dye. The sebaceous gland can serve as a reservoir for the dye. The higher concentration of the dye within the sebaceous gland can provide the dye diffusion in depth of the hair follicle and skin. At the level of isthmus where IRS is desintegrated and keratinized ORS makes only a few cells layer, the dye can diffuse in surrounding tissue and in depth more effectively then through the stratum corneum on the skin surface.

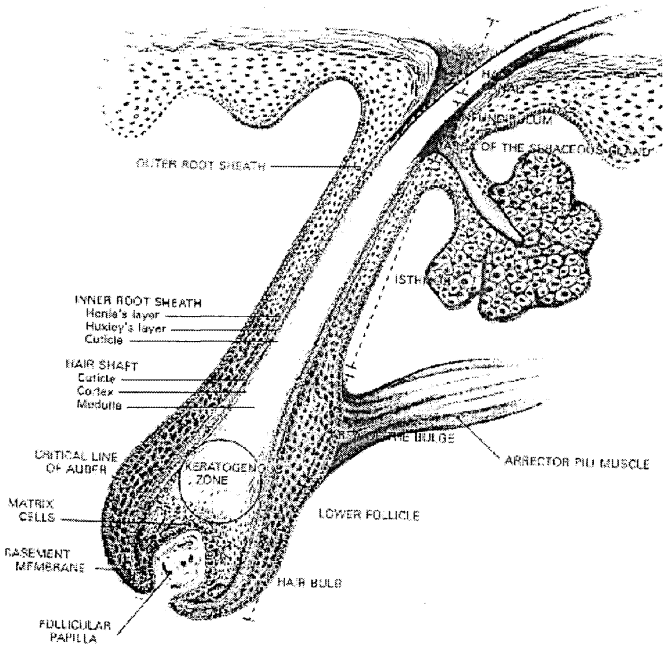

Figure 1. Anatomy of the hair follicle (anagen stage)[13].

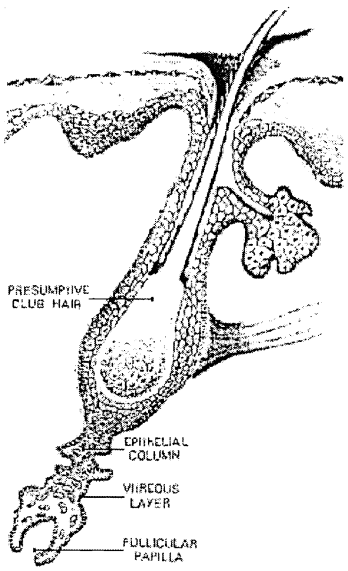

Figure 2. Anatomy of the hair follicle (catagen stage).[13]

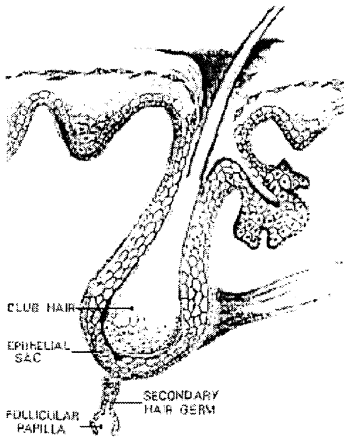

Figure 3. Anatomy of the hair follicle (telogen stage).[13]

A telogen hair is distinguished by its fully keratinized club, which is surrounded by an epithelial sac (Fig. 3).[12,13] Hair follicle length in telogen stage decreases to about 1 mm.

Transitive stage between anagen and telogen is catagen stage (Fig. 2). Catagen follicles have a several anatomic features that are peculiar to this phase of the life cycle. Above the papilla, the follicular epithelium remains as a thin column of pale-staining, nonpigmented, undifferentiated cells.[12,13] Length of hair follicle in catagen stage varies from 1 to 1.5 mm.

3. METHODS AND MATERIALS

We used the following commercially available USA haircolors: "Clairol Loving Care" (83 Natural black), Grecian-5 (Jet black), "Just for men" (Jet black), "L'Oreal Feria" (21 Natural black), "Clairol Hydrience" (54 Soft Black), and the following food dyes: Food Blue (Russia, from confectionery), Indigo Carmine, Fast Green (Sigma Chemical Co., USA), Erioglaucine (Aldrich Chemical Co., USA), and biological dyes: Toluidine Blue, Methylene Blue (Sigma Chemical Co., USA), Indocyanine Green (Aldrich Chemical Co., USA), and Brilliant Green (Russia, Tver' Pharmaceutical Fabric).

Absorption spectra of all haircolors and dyes in the 400-1500 nm wavelength range were recorded by the spectrophotometer with the integrating sphere CARY-2415 (Switzerland). Haircolors were prepared according to instruction of company enclosed to wrapper.

Spectra of individual hairs dyed by haircolors were recorded. The hair holder was built using the spectrometer's slit with a manually controlled width. The hair under investigation was carefully displaced between stainless edges of the slit. The intensity of the transmitted light trough aligned slit with and without a hair shaft was measured. The hair shaft transmittance was calculated as a ratio of these two measurements for each wavelength.

Various lotions were used as solvents of food and biological dyes. Concentration of all dyes except Food Blue was made 1 mg/ml. Concentration of this dye was 10 mg/ml. To record spectra 100-μm cuvette was used.

The human skin test sites were 5×5 cm rectangular in size. Pre-operative preparation included the treatment of the site by ethanol or hot compress in 10 minutes. Then the warm dyeing solution was spread on the skin surface. For increasing of dye diffusion into hair follicles we applied the follows methods: massage and/or heat. We used heater or massager with electrical heat. In the first case we sheeted the place with polyethylene film and applied the heater (up to 45 ^{0}C). In the second case the skin surface was massaged at the same temperature by electrical massager. The time of procedure was 15-20 min. The time of dyeing by haircolors was chosen in accordance to instruction of company. Then the dyeing solution was removed by cotton wool tampon impregnated by warm water.

Hair samples were collected from exterior side of forearms and lower legs of 6 healthy volunteers with fair or brown hairs. Hairs were epilated together with hair follicles by cosmetic tweezers. Hairs were epilated just after dyeing and after 15 min, 30 min, 1 h, 2 h, and 5 h after dyeing.

Skin biopsy was done by standard method with the use of biopsy punch (Acuderm inc., USA) from exterior side of forearm of 2 healthy volunteers with brown hairs. Skin with growing hair was chosen for biopsy. Size of tissue sample was 2 mm in diameter and 4-5 mm in depth. After excision the tissue sample was put between two glass plates and kept under mechanical pressure during a few hours to receive dry and optically transparent sample. After drying the samples were put into immersion oil.

For *in vitro* study we used the human skin samples with hairs after 24 hours *post mortem.* Skin was dyed by Palomar ICG-lotion during 30 min. Dyed human skin biopsy sample were taken using the same procedure as for *in vivo* biopsy.

Optical imaging microscopy was used for investigation of hair and biopsy samples. Photos of all samples were made by the system composed of a video-microscope (VHS color camera Panasonic NV-RX70EN and microscope objective) interfaced with a personal computer.

The dyeing of the human skin was also investigated. Skin surface was dyed by Palomar ICG-lotions (lotions with Indocyanine Green) then the lotion was deleted thoroughly. A few skin strips were done by adhesive tape (Multi-Film, TESA, Beiersdorf, Hamburg) from one place on inner side of forearm of two volunteers and stuck on subject glasses.

Spectra of total transmission were recorded by the spectrophotometer CARY-2415. As a reference a glass with tape was used.

4. RESULTS AND DISCUSSION

Usually ruby (694 nm), diode (800-830 nm) and YAG:Nd (1064 nm) laser irradiation is used for damage of hair follicles.[1-11] The main natural absorber is melanin in hair shaft and follicular papilla. To make more selectivity dyes with absorption bands at these wavelengths should be used. Analyzed haircolors have no sharp absorption bands in the visual and NIR. The typical total transmission spectra of individual hair shafts of undyed hair and two samples of dyed by haircolors are shown in Fig. 4. It is well seen that optical density of hair shaft is decreased with increasing of the wavelength. In NIR optical density of dyed and undyed hair shaft is vary small. Moreover, dyed hair shaft can has less optical density then undyed. That happens because scattering is very important for light transmittance in the NIR. Applying of commercial haircolors containing hair conditioners allows for reduction of superficial scattering and therefore for reduction of the optical density.

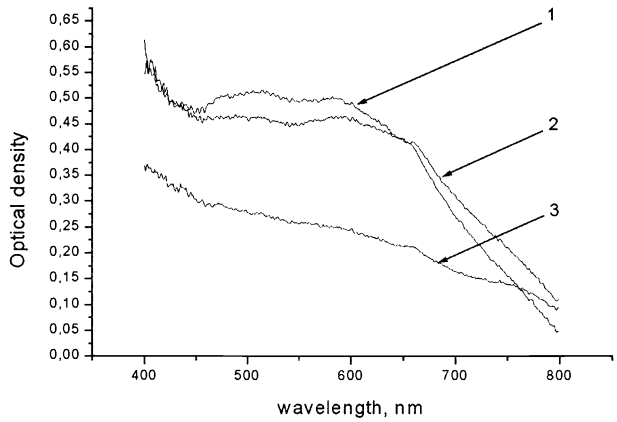

Figure 4. Optical density of hairs dyed by black haircolors "Clairol Loving Care" (1), "Grecian-5" (2), and undyed hair (3) recorded by a spectrophotometer.

Absorption spectra of studied dyes dissolved in glycerol are shown in the Figs. 5 and 6. It is well seen that the following dyes have the greatest absorption coefficient: Methylene Blue (650 cm^{-1} at 665 nm), Erioglaucine (653 cm^{-1} at 634 nm), and Indocyanine Green (756 cm^{-1} at 790 nm). Food Blue has a broad absorption band with peak 260 cm^{-1} at 620 nm. These dyes can be suitable for ruby (649 nm) or diode (800 nm) lasers. The analyzed dyes do not have absorption peaks near 1064 nm.

For *in vivo* measurements we have chosen Indocyanine Green (ICG) as a dye having the greatest absorption coefficient at 800 nm. ICG has chemical formula $C_{43}H_{47}N_2O_6S_2Na$ and molecular weight 775. It is tricarbocyanine type of dye with infrared absorbing properties. It has little or no absorption in the visible. It is used as a diagnostic aid for blood volume determination, cardiac output, or hepatic function. The principal advantages causing the rapid acceptance of the dye in medical practice were the presence of an 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. [14,15]

In Fig. 7 absorption spectra of ICG in different solvents and two Palomar ICG lotions are shown. We can see that in water and Aloe Vera Gel (based on water) the ICG peak is suppressed for the chosen ICG concentration.[14] IR peak can be seen in water solutions for the less concentrations of ICG. Extremely high absorption above 1000 cm^{-1} has Palomar ICG lotion-1. Very remarkable that this lotion has 1.8-fold higher absorption coefficient than ICG dissolved in any of solvents at the same concentration of ICG. Absorption coefficients of Palomar ICG lotion-1 and lotion-2 are 1037 and 540 cm^{-1} at 789 nm, respectively.

Two Palomar Medical Products, Inc. ICG lotions were tested: Palomar ICG lotion-1 and –2. Figures 8 and 9 illustrate *in vivo* dye diffusion into the human skin. In Fig. 8 follicle in telogen stage is shown. For calibration of linear sizes of color images a precise testing object (scale) shown in the images was used. The skin was dyed by Palomar ICG lotion-1 with use of massage and heat. The skin surface is well seen. It corresponds to upper dyed part of epilated hair (approximately 0.3 mm, which is dyed sebaceous discharge attached to the hair shaft) in Fig. 8. Sebaceous discharge filling of the hair canal (see Figs. 1-3) is well dyed due to dye interaction with lipids. Then part of hair without any dye follows (about 0.5 mm), because the dye does not diffuse into dense keratinized tissue of a hair shaft. The dyeing lotion diffuses along of hair shaft to soft nonkeratinized tissues. The depth of dyeing is approximately 0.2 mm. Figure 9 shows biopsy sample with the hair in anagen stage. Distance from the surface of the skin to the dyed portion of a follicle is differed for hairs in anagen, catagen, and telogen stages. It is about 0.3 mm and 0.8 mm for anagen and telogen hairs, respectively. In catagen stage this distance has transitive value.

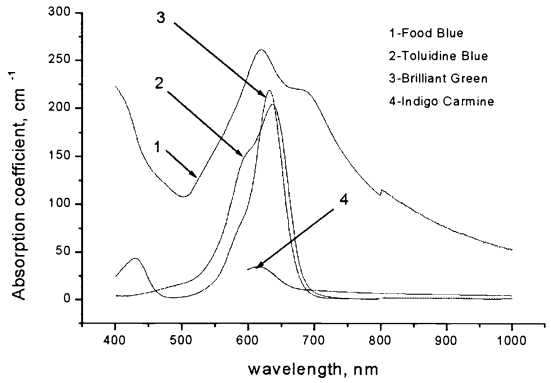

Figure 5. Absorption spectra of dye solutions in glycerol with concentrations 10 mg/ml (1 – Food Blue) and with concentration 1 mg/ml (2 – Toluidine Blue, 3 – Brilliant Green, 5 – Indigo Carmine) recorded by a spectrophotometer.

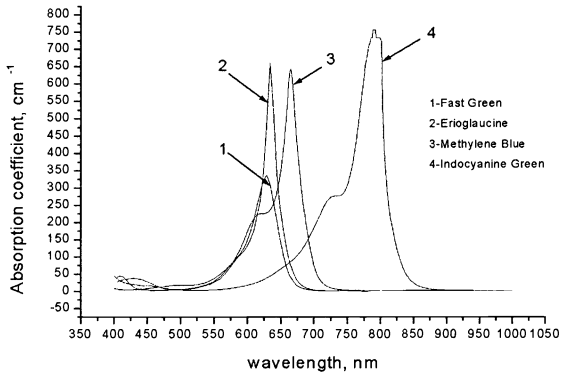

Figure 6. Absorption spectra of dye solutions in glycerol with concentrations 1 mg/ml recorded by a spectrophotometer. 1 – Fast Green, 2 – Erioglaucine, 3 – Methylene Blue, 4 – Indocyanine Green.

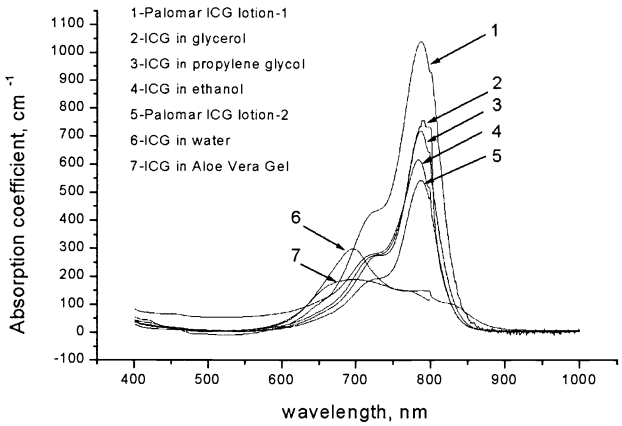

Figure 7. Absorption spectra of Indocyanine Green in different solvents with concentration of 1 mg/ml recorded by a spectrophotometer. 1 – Palomar ICG lotion-1, 2 – ICG in glycerol, 3 – ICG in propylene glycol, 4 – ICG in ethanol, 5 – Palomar ICG lotion-2, 6 – ICG in water, 7 – ICG in Aloe Vera Gel.

Some understanding of presented results follows from the hair follicle and surrounding tissues structure for various stages of a life cycle (see Figs. 1-3).[13] The follicle is bounded by a basement membrane ("glassy membrane"). The outer root sheath

(ORS) is the most peripheral of the cellular compartments. The permanent portion of the follicle begins with the hair canal region, extending from the skin surface to the level of the epidermal-dermal junction. Its lower part later becomes the "infundibular unit." That means, the region between epidermal-dermal junction (opened to the skin surface) and intraepidermal infundibulum, is the path for lotion penetration, because only a few cell layers of living cells of epidermis and the basement membrane (very thin) separate hair canal, filled with lotion, from dermis, which is due its fibrous structure (collagen fibers) should be enough permeable for the lotion. So, dermis around ORS, ORS itself, as a living cell structure, can be dyed. Actually, in the experiments sometimes the dyed area around the hair shaft was about twice bigger than diameter of the hair shaft. But usually the dyed area around the hair shaft was only slightly bigger than diameter of the hair shaft, so ICG does not penetrate deeply within dermis, but the living cells of the inner and outer root sheaths always were dyed. The transient portion of the follicle begins at the area of the bulge and extends to the deepest levels of the follicle. The epilated hair contains: whole follicle, including ORS as an outmost part of epilated follicle. The upper part of follicle is dyed therefore ORS cells are dyed. The basement membrane and dermis around hair follicle for some hairs also can be dyed.

The inner root sheath (IRS), which normally disintegrates in the anagen follicle at the level of the sebaceous duct, is totally absent for the telogen follicle, that means that with forming of hair club keratinized portion of a hair follicle increases and a gap (cannel) between hair shaft and keratinized ORS is formed, i.e., dyeing substance diffuses dipper. Therefore, the penetration depth of the lotion for catagen and telogen hairs should be higher (as a distance from the skin surface). It is well seen from data in Figs 2,3 and 8. Very important to say that the upper part of hair follicle, so called permanent portion, especially area around sebaceous gland and even isthmus, are always dyed for hairs in any phase of their growing (catagen, telogen, or anagen).

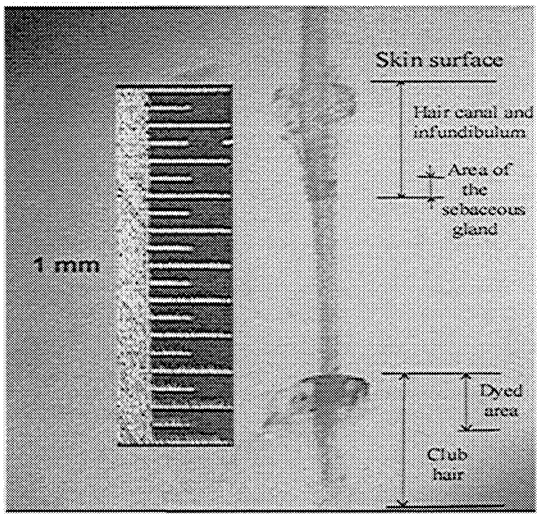

Figure 8. Hair in telogen stage dyed by Palomar ICG lotion-1 epilated from the forearm of volunteer just after dyeing.

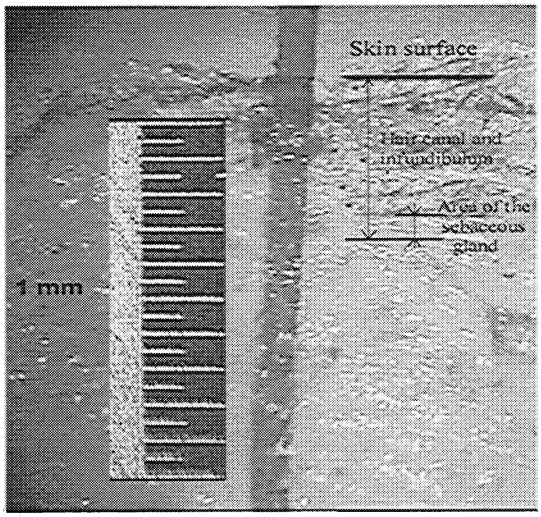

Figure 9. Hair in anagen stage, biopsy of the skin from the forearm of volunteer just after dyeing by Palomar ICG lotion-1.

In vivo studies of diffusion of Food Blue dissolved in glycerol in hair follicles showed the depth of dyeing of about 1 mm for hairs in telogen stage and about 0.5 mm for hairs in anagen stage.

For 44 hair samples (25 in anagen stage and 19 in telogen or catagen stages) the mean value of dyed area of follicle in all stages was 0.3±0.1 mm. Mean distance from the skin surface to dyed portion of follicle was 0.3±0.1 for anagen hairs and 0.7±0.1 for telogen or catagen hairs. Thus the mean depth of dyed area of follicle in telogen and catagen stages is about 1 mm from the skin surface. For anagen hairs the mean depth is about 0.6 mm.

Method of dyeing includes massage with heat in 15-20 min. Hairs from dyed place were removed just after dyeing and after 15 min, 30 min, 1 h, 2 h, and 5 h after dyeing. The most intense dyeing of hair follicles was just after procedure. Then it diffuses in ambient tissue. Cleaning of skin tissue thoroughly is important to eliminate overheat of skin surface.

Interaction of Palomar ICG lotion-1 and -2 with the skin layers was investigated by a tape stripping method.[16] Skin strips of 3-5 μm of thickness were taken from two volunteers. Figure 10 shows optical spectra of the first and the second strips of

skin dyed by Palomar ICG lotion-1 and spectrum of Palomar ICG lotion-1. Figure 11 shows optical spectra of the first and the second strips of skin dyed by Palomar ICG lotion-2 and spectrum of Palomar ICG lotion-2.

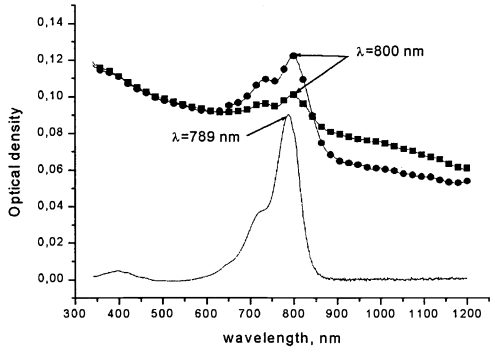

Figure 10. Optical spectra of Palomar ICG lotion-1 (solid curve, optical density is divided by 5) and two first strips (circles – the first strip, squares – the second strip) obtained from the skin dyed by this lotion.

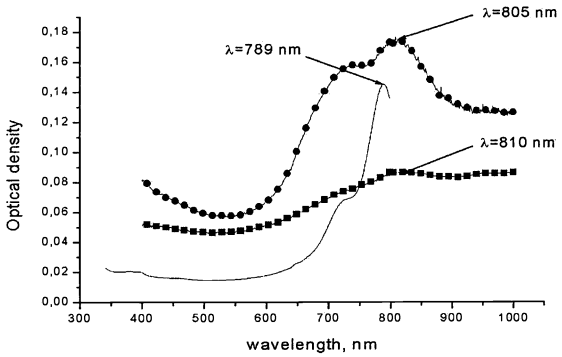

Figure 11. Optical spectra of Palomar ICG lotion-2 (solid curve, optical density is divided by 5) and two strips (circles – the first strip, squares – the second strip) obtained from the skin dyed by this lotion.

Shift to the longer wavelengths of about 11 nm (for the first and the second strips of the skin treated by Palomar ICG-lotion-1), 16 nm (for the first strip of the skin treated by Palomar ICG lotion-2), and 21 nm (for the second strip of the skin treated by Palomar ICG lotion-2) for the main absorption peak of ICG in the stratum corneum was defined. Such shift is caused by ICG binding with organic molecules of a living tissue and will provide more precise correspondence of acting laser radiation and dyed tissue target. It was reported in a few papers that due to binding with cell proteins the IR peak of ICG is moved to the longer wavelengths: to 805 nm for the blood[17] and human skin *in vivo,*[18] to 810 nm for epidermal cell cultures.[19] In present study for the stratum corneum of the human skin (*in vitro* measurements of *in vivo* dyed skin) peak was moved to 800 nm (Fig. 10) and to 805 and 810 nm (Fig. 11). In our and Ref. 18 measurements the influence of a free ICG component can be important, because nothing was done to wash out free ICG, which IR peak is observed at 789-790 nm. In Ref. 19 authors carefully washed out free ICG from the studied samples that is why they exclude the influence of free ICG and received pure spectrum of the bounded ICG at 810 nm. Spectra for Palomar ICG lotion-2 (Fig.11) well demonstrate that at the skin surface (first strip) we have some mix of free and bounded ICG (shift is 805 nm) and at the depth of 3-5 μm (second strip) all ICG is bounded (shift is 810 nm, well corresponds to cell culture measurements of Ref. 19).

In comparison with a living tissue the ICG distributions in *post mortem* dyed skin samples are more diffusive and not so deep (the depth is not more than 0.7 mm). The transverse diffusion of dye around the hair is much bigger (area around is up to 0.5 mm) than for living tissue and can be explained by some degradation of cells and basement membrane of hair follicle, but still dye penetrates through the hair canal and sebaceous gland.

5. CONCLUSION

In this study we have investigated some haircolors and dyes (food and biological) for potential using in laser selective thermolysis. Absorption coefficients of all dyes were obtained. Safe for humans and having the greatest absorbency at 800-810 nm is Indocyanine Green dye. Suitable for use of ruby laser (649 nm) dyes are Erioglaucine, Methylene Blue, Toluidine Blue, Fast Green, and Food Blue.

We have developed ICG lotions and method of dyeing and tested their *in vivo*. Using the designed ICG-lotions for *in vivo* dyeing of the human skin surface and studying of epilated and taken by biopsy hairs follicles it was shown that in dependence of hair growing stage the ICG penetration depth is in the range from 0.6 to 1 mm.

ICG penetrates into follicle through the hair canal and sebaceous gland serves as a reservoir for dye penetration in depth, the dye is mainly distributed within a small area around the hair shaft, which includes the inner and outer root sheaths cells, that

should provide a high concentration of dye in the area around the hair shaft, i.e., more effective photothermolysis under 800 nm diode laser irradiation.

Shift on 11-21 nm of absorption peak of ICG to the longer wavelengths due to ICG binding with cell proteins in the human skin was found.

In comparison with a living tissue the ICG distributions in *post mortem* dyed skin samples are more diffusive and not so deep (the depth is not more than 0.7 mm). The transverse diffusion of dye around the hair is much bigger (area around is up to 0.5 mm) than for living tissue and can be explained by some degradation of cells and basement membrane of hair follicle.

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