SOLID LIPID CURCUMIN-LOADED PARTICLES FOR IN VIVO FLUORESCENT IMAGING IN HUMANS:

A PROOF OF CONCEPT

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Abstract - The possibility to use oral intake of fluorescent dyes approved on humans for intravital fluorescence *in vivo* imaging is attractive due to its simplicity and non-invasive character. Here we investigate the potential of solid lipid curcumin particles (SLCP) for imaging of capillary permeability using fluorescence video capillaroscopy. Curcumin fluorescence corresponding to 0.7 μg/ml concentration was observed from blood plasma after oral consumption of 2 g of SLCP. However, this signal was ~15% higher than that of blood plasma intrinsic fluorescence, which was itself at least 10-fold lower than the background signal from pericapillary tissue. By comparing the optical properties and concentration to the case of intravenous injection of sodium fluorescein, we analyzed which parameters of the experiment should be optimized for using oral consumption of fluorescing compounds in intravital *in vivo* capillaroscopy measurements.

INTRODUCTION

Current trends in biomedical diagnostics include the development of the molecular imaging, i.e. visualization of certain types of molecules in the body. Molecular imaging allows investigating biochemical processes in the organism at the molecular level and can be performed using a number of techniques, including PET, MRI etc. However, for research purposes optical methods appear to be the most useful. Being capable of providing subcellular resolution, optical techniques such as confocal fluorescence microscopy with exogenous labels have substantially

advanced the understanding of molecular processes *in vitro* (in cell cultures) and *in vivo*, mostly on the laboratory animals. The development of molecular imaging methods is closely connected with creation of advanced probes, which can be used for selective and efficient labeling of predetermined molecules and can be detected with a high contrast. Typical types of labels include genetically encoded labels, mostly fluorescent proteins-based, labeled antibodies, as well as various nanoparticles. Obviously, not all exogenous labels, which are perfectly suited for experiments on laboratory animals, can be applied for humans – the main limitation here is their adverse and largely unknown influence on the human organism [1]. Hence, only a few substances are approved for use as probes on humans, of which fluorescent dyes are probably the most well-known [2]. Generally, intravenous injection or topical application does not allow to selectively label certain molecules, however, its enhanced accumulation in tissues due to specific mechanisms can be used for biomedical diagnostics, e.g. for cancer diagnostics or even treatment (in the case of photodynamic therapy).

Application of fluorescent dyes also allows for monitoring of processes, which can be hardly investigated with endogenous contrast or other methods. This can be illustrated by the example of studies, which focused on increased permeability of blood capillaries accompanying several diseases [3, 4, 5]. After intravenous injection of fluorescein, the area of nailfold was examined in vivo using fluorescence microscopy, and the kinetic parameters of fluorescing halo around the capillaries, formed as the result of blood plasma leakage from the vessels, were investigated. This method allowed investigation of transcapillary diffusion and fluid exchange and could be promising for diagnostic in cardiology and dermatology, however, its applicability is reduced due to the necessity of invasive intravenous injection of dye.

Currently, different containers for efficient drug delivery into the blood stream via oral consumption are being developed. For instance, several formulations exist for oral delivery of curcumin, a natural diphenolic compound derived from turmeric *Curcuma longa*. The interest to curcumin is stimulated by extensive data reported in the literature indicating anticancer and anti-neurodegenerative potential of curcumin [6]. The evidence base for these properties of curcumin has been multiply reviewed, and ~200 clinical trials focusing at the positive effects of curcumin have been performed [7]. The key factor for curcumin bioactivity is its concentration in blood, and it was shown that oral intake of pure curcumin or curcumin mixed with lipids is not enough, thus stimulating the development of delivery methods [8]. The use of solid lipid curcumin particles (SLCP) was reported to overcome this limitation and to facilitate curcumin delivery into blood in concentrations necessary for the clinical effect. On the other hand, curcumin represents a natural fluorophore exhibiting fluorescence emission in the green spectral region with an acceptable quantum yield values ranging from ~0.01 to ~0.2 for most solvents [9]. Thus,

accumulation of curcumin in blood could be potentially use for its fluorescent tracing. Importantly, a recent work demonstrated the possibility of Alzheimer disease diagnosis using fluorescence of amyloid plaques in the human retina: amyloid bodies “light up” due to selective binding of curcumin delivered via SLCP consumption [10]. Collectively, these facts motivate us to investigate the SLCP suitability for intravital imaging.

MATERIALS AND METHODS

Materials

SLCP were purchased from Nutrivene (supplied under the trademark LongVida). Bovine serum albumin (BSA) was purchased from Sigma (Germany).

The blood samples were taken from a healthy volunteer in a 5 ml EDTA containers before and one hour [11] after SLCP consumption. Four 500 mg SLCP capsules were taken simultaneously. The volunteer fastened overnight. Blood was immediately centrifuged at 1600 rpm during 10 minutes to obtain blood plasma. To measure fluorescence spectra, blood plasma was diluted with bidistilled water with dilution ratio of 1:40 (plasma-:-water). We note that the spectra of blood plasma presented in this paper are shown after renormalization, which takes into account the dilution coefficients.

Methods

Fluorescence spectra were recorded using the FluoroMax-4 fluorometer (Horiba, Japan), the slits spectral width was set to 5 nm. Excitation/registration wavelengths: 430 nm / 450-700 nm. Absorption spectra were measured using the Lambda-25 spectrophotometer (Perkin-Elmer, USA).

Fluorescence microscopy measurements were carried out using a Nikon Eclipse Ni-E microscope with a 20x objective (NA 0.75, working distance 0.35 mm). As an excitation source, mercury lamp Nikon Intensilight C-HFGIE with the system of dichroic filters was used. The fluorescence signal was collected in epi-configuration mode and was detected by CCD camera. Excitation/registration wavelengths were the following: 375 ± 15 nm / 460 ± 30 nm, 480 ± 15 nm / 535 ± 20 nm. Typical exposure values were 100 ms for observing the fluorescence signal of the skin tissue *in vivo* and blood *in vitro*, and 2-4 s for observing plasma fluorescence *in vitro*.

RESULTS

To determine the possibility of detecting the fluorescent signal from blood plasma *in vivo* using wide field microscopy, we performed a series of observations of nailfold microcapillaries on human volunteers. Fluorescence imaging performed at two excitation wavelength (375 and

480 nm) revealed the presence of emission signal around the capillaries, which appeared dark due to light absorption by hemoglobin (Figure 1). The origin of fluorescence signal from the area of the nailfold was studied in detail in [12] using in vivo two-photon microscopy. It was shown that blood capillaries are located in the finger-like dermal papillae, composed of elastin and collagen types I and III [13]. The area around the capillary is shown in Figure 1 with dashed line (perivascular zone) corresponds to the viable epidermis. Generally, fluorescence signal observed using wide field microscopy is originated from collagen, keratin and elastin molecules, as well as from the epidermal cells keratinocytes. Blood plasma leakage from the capillaries could potentially alter fluorescence signal, both because of its intrinsic fluorescence [14] and the presence of exogenous fluorescence dye – in our case, curcumin. In some cases intense fluorescence signal also can be observed from red blood cells from hemoglobin degradation product [15].

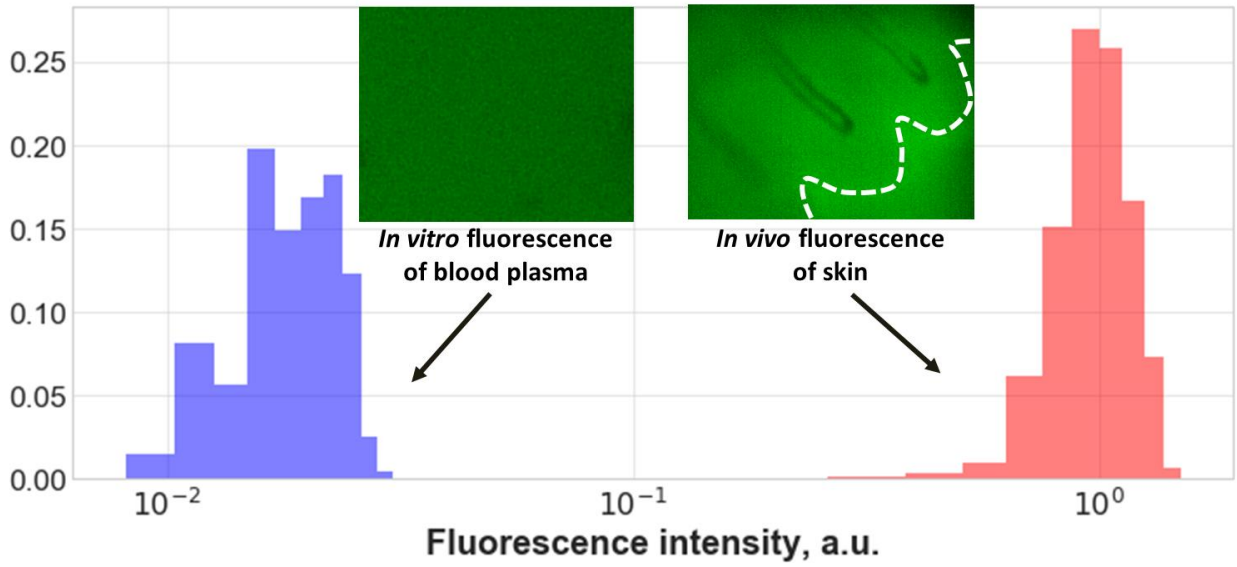

Figure 1. Distribution of fluorescence intensity (480 ± 15 nm/535 ± 20 nm) for the blood plasma sample and nailfold area. Detection volume and acquisition parameters were similar in both cases. The inset presents typical fluorescence images obtained from blood plasma (left) and nailbed area (right). Perivascular zone is indicated with the dashed line.

We observed that fluorescence intensity of tissues located near the microvessels (perivascular zone and horny layer of the skin) obtained *in vivo* is approximately 10-20 times higher in comparison to fluorescence signal of blood plasma obtained *in vitro* with the same excitation and detection parameters. Thus, detection of the fluorescence signal from the blood plasma, extravasated from capillaries as a result of transcapillary diffusion, is hardly detectable using wide field fluorescence microscopy and exogenous labels are required to investigate this process. Hence, as the next step we quantified the impact of curcumin fluorescence to blood plasma emission signal after SLCP consumption.

Absorption and fluorescence emission spectra of curcumin are presented in Figure 2A. As curcumin is poorly soluble in water, the experiments were performed in ethanol solution. Gota et al. [11] made use of high performance liquid chromatography (HPLC) to quantify free curcumin concentration in blood plasma samples after SLCP consumption, and it was shown that the maximum value was ~20 ng/ml. Figure 2B demonstrates fluorescence emission of the blood plasma *in vitro* sample and of 20 ng/ml curcumin solution in water. As can be seen, at this concentration curcumin fluorescence is significantly lower than the background signal originated from endogenous fluorophores contained in blood plasma.

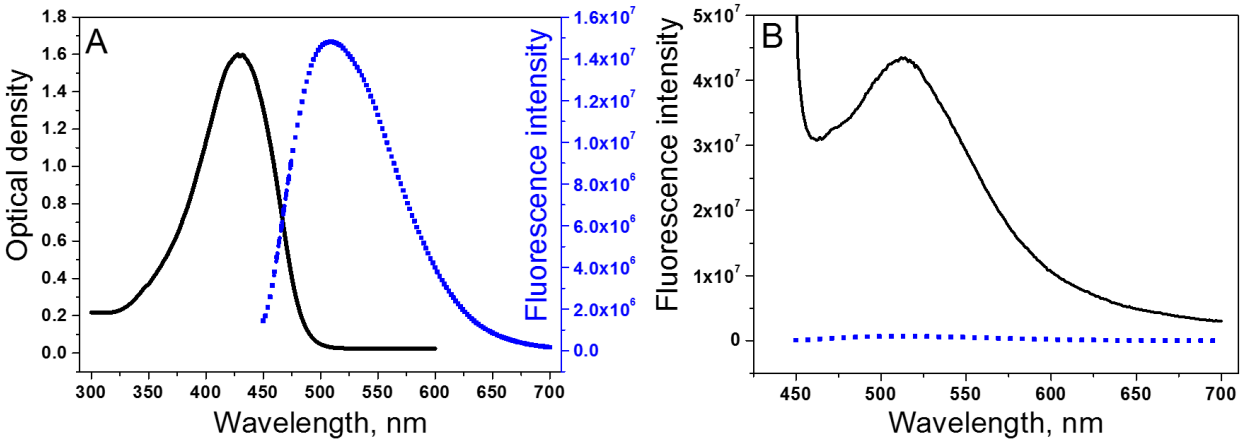

Figure 2. A) Absorption (30 μM, black line) and fluorescence emission (1 μM, blue dotted line) spectra of curcumin in ethanol. B) Fluorescence spectra of blood plasma (black line) and 20 ng/ml curcumin solution in water (blue dotted line). Excitation was performed at 430 nm wavelength, which corresponds to the maximum of curcumin absorption spectrum.

However, the authors [11] quantified the concentration of free curcumin molecules, i.e. only the signal at retention time corresponding to free curcumin was assessed. This is due to the fact that only free curcumin is supposed to exhibit necessary biological effect, while its products are not crucial for this. At the same time, being a hydrophobic molecule, curcumin can be expected to form complexes with components of blood, including albumin – the main transport protein of blood plasma. Indeed, it is known from the literature data that albumin exhibits a micromolar affinity towards curcumin [16], that, together with high concentration of albumin in blood plasma (~1 mM) suggests that binding should be very effective.

To illustrate the interaction process between albumin and curcumin, we performed fluorescence titration of curcumin solution with bovine serum albumin (BSA), which is a close analog of human serum albumin in terms of ligand binding. Figure 3A demonstrates changes of curcumin fluorescence spectrum in the presence of different BSA concentrations. It can be seen that both blue shift of the emission spectrum and fluorescence enhancement are observed when BSA concentration increases, indicating complex formation.

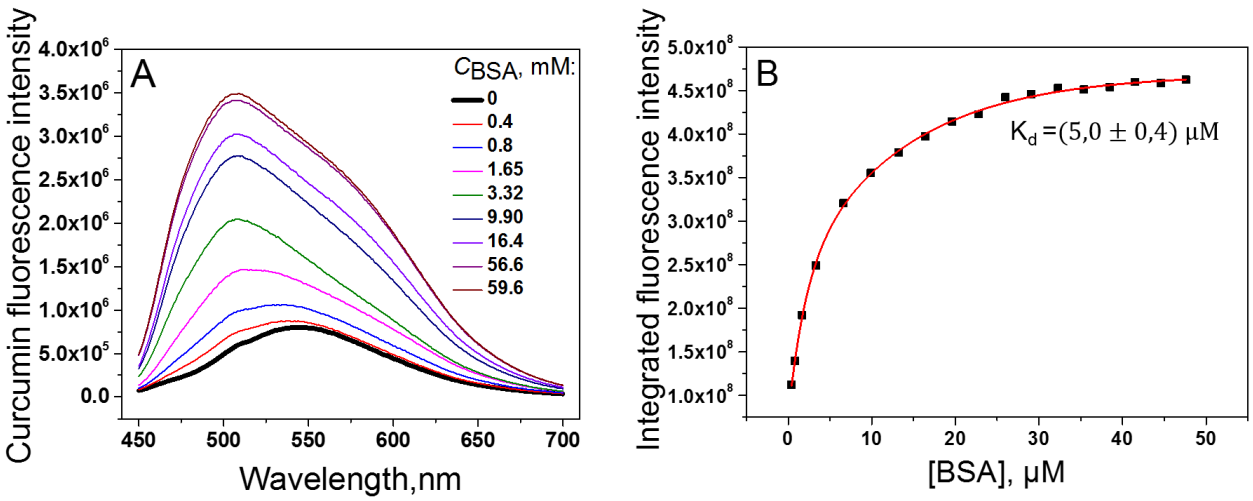

Figure 3. A) Fluorescence spectra of curcumin solution (1 μM) in the presence of BSA at different concentrations. Excitation wavelength was set to 430 nm. B) The dependence of integral fluorescence intensity of curcumin on BSA concentration and its fit to the 1:1 complex model with the dissociation constant K_d = 5 μM.

Fitting of the titration curve, which represents the saturating dependence of curcumin fluorescence intensity on BSA concentration (Figure 3B), yielded the 5 μM affinity constant, which is in a good agreement with the literature data obtained for BSA [16] and HSA [17, 18, 19].

Assuming that not only free, but also bound curcumin, as well as its complexes, may exhibit fluorescence signal in blood plasma and result in enhanced fluorescence intensity, we performed the measurements on a blood plasma of a volunteer before and 1 hour after consumption of SLCP.

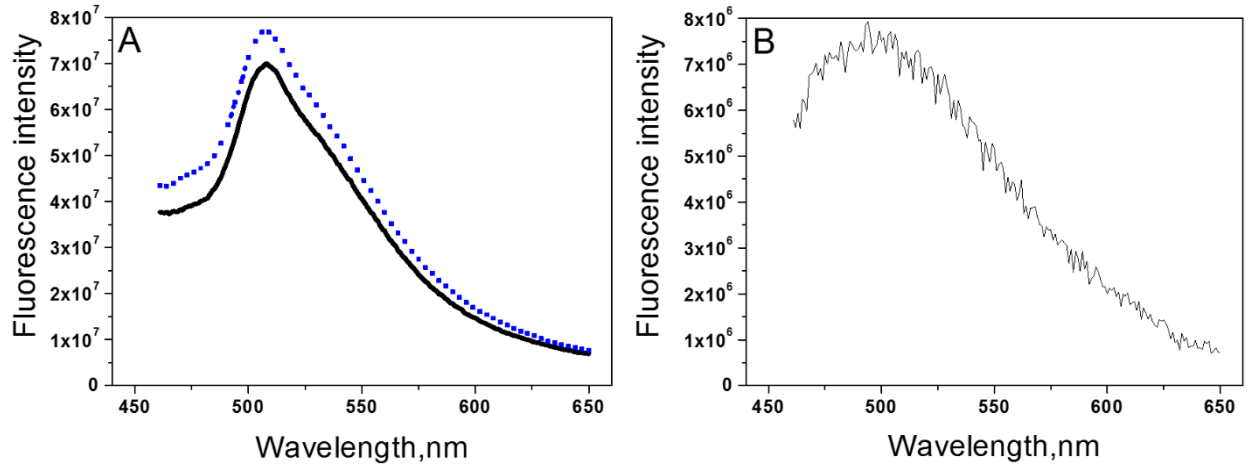

Figure 4. A) Fluorescence spectra of blood plasma of a volunteer before (black line) and 1 hour after (blue dotted line) SLCP oral consumption. B) The difference spectrum, corresponding to curcumin fluorescence.

As can be seen from the Figure 4A, in this case the increase in fluorescence after SLCP consumption was quite low (~15%) considering the fact that a signal at least 10-fold higher than intrinsic fluorescence of blood plasma is required to be detected using wide field microscopy. The position of maximum of the difference spectrum is located at ca 500 nm, indicating that curcumin is predominantly bound (see Fig. 3A). By using the calibration curve obtained for the BSA-curcumin complexes, it can be estimated that the overall curcumin concentration as revealed from the fluorescence measurements is ~0.7 μg/ml, which is of the same order of magnitude as the estimation obtained by affinity constant and is 35 times higher than the concentration of free curcumin in blood plasma obtained using HPLC [11].

DISCUSSION

The possibility to use oral intake of fluorescent dyes approved on humans for intravital fluorescence imaging is attractive due to its simplicity and non-invasive character. In this context, curcumin-loaded formulations, which are designed to deliver it to blood, are of interest. In this work we observed that, indeed, the increase in blood plasma fluorescence can be observed after oral consumption of SLCP, and the difference spectrum is close to that of bound curcumin. However, the observed increase of signal over the background is ~15%. *In vivo* experiments performed on the human nailbed demonstrated the presence of significant fluorescent signal around the capillaries, which originated from structural proteins and cells of epidermis. As this signal is at least 10-fold higher than the signal of blood plasma (at similar acquisition parameters and detection volume), one can expect blood plasma extravasation to be observable is the signal of exogenous dye is, correspondingly, 10 times higher than that of intrinsic blood plasma fluorescence. Our data demonstrates that this is not the case for SLCP consumption. While the accumulated curcumin concentrations occurred to be enough for fluorescent imaging of retinal amyloid pathology [10], is was insufficient for skin investigation with wide field microscopy. Presumably, this limitation can be overcome by using more sophisticated methods like confocal laser scanning microscopy or two-photon microscopy, which may help to reduce the background signal and focusing on target region. Moreover, the use of fluorescence photobleaching can reduce the background signal of the skin and increase detection limit of target substances [20]. Another way to both improve fluorescent contrast of plasma to skin fluorescence and reduce light scattering from tissues is the use of optical clearing agents [21, 22]. Also the signal to background ratio can be increased by using dyes with red shifted emission, e.g. indocyanine green [23]. This will help to reduce both backgrounds from blood plasma and tissues surrounding capillaries.

It is interesting to compare the system parameters obtained in this this work with that used for fluorescence videocapillaroscopy after intravenous dye (sodium fluorescein) injection (Table 1).

Table 1. Comparison of photophysical parameters and concentration of BSA-bound curcumin (in this work) and sodium fluorescein [3].

	Excitation/emission, nm	Quantum yield	Solubility in water	Concentration
Curcumin (BSA-bound)	430/510	0.05 [16]	Low solubility	0.7 μg/ml
Na-fluorescein	480/510	0.9 [24]	Soluble	40 μg/ml

For instance, in [3] 20% sodium fluorescein was used to reach concentrations of 0.2 ml/l in blood, corresponding to ca 0.1 mM or 40 μg/ml, i.e. ~50 times higher than concentration detected after SLCP consumption in this work. Moreover, sodium fluorescein exhibits higher fluorescence quantum yield compared to curcumin (0.9 vs 0.05, respectively, see Table 1), and can be excited at longer wavelength (480 nm vs 430 nm, respectively), thus reducing the background signal. Collectively, we estimate that in the case of sodium fluorescein intravenous injection the signal to background ratio was 1000-fold higher than for SLCP in this work.

CONCLUSION

The fluorescent label, which can be used after oral consumption of approved compound, is highly desirable for intravital imaging, e.g. for *in vivo* studying of microcirculation processes. The key factors affecting applicability of this approach are label concentration in blood, its fluorescence quantum yield and spectral parameters. In this work we have tested the possibility of FDA-approved curcumin containers, SLCP, for imaging of fluid extravasation from blood vessels. We observed that when using wide field microscopy, currently the fluorescence signal from curcumin is less than the intrinsic fluorescence of blood plasma, while the fluorescence of blood plasma itself is lower than the background signal from tissues surrounding capillaries. The possible ways to increase the signal to background ratio include the use of confocal microscopy, dyes with red shifted emission and higher quantum yield. The approach used in this work can be applied for determination of content of fluorescent dye in blood that may help in optimization of containers for delivery of concentrations sufficient for intravital *in vivo* imaging. Moreover, distribution of topically applied curcumin can be assessed by the used approach.

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REFERENCES

1. R. Alford, H. M. Simpson, J. Duberman, G. C. Hill, M. Ogawa, C. Regino, and P. L. Choyke, Mol. Imaging **8** (6), 7290-2009 (2009).
2. T. Robertson, F. Bunel, and M. Roberts, Cells **2** (3), 591-606 (2013).
3. A. Bollinger, K. Jäger, A. Roten, C. Timeus, and F. Mahler, Pflug. Arch. Eur. J. Phy. **382** (2), 137-143 (1979).
4. A. Bollinger, U. K. Franzeck, and K. Jäger, *Vasomotion and Quantitative Capillaroscopy* (Karger Publishers, Konstanz, 1983).
5. A. Boilinger, I. Herrig, M. Fischer, U. Hoffmann, and U. K. Franzeck, Int. J. Microcirc. **15** (Suppl. 1), 41-44 (1995).
6. G. Mazzanti and S. Di Giacomo, Molecules **21** (9), 1243 (2016).
7. U.S. National Library of Medicine, http://clinicaltrials.gov/ct2/results?term=curcumin.
8. M. M. Yallapu and M. Jaggi, S. C. Chauhan, Drug Discov. Today 17 (1-2), 71-80 (2012).
9. K. I. Priyadarsini, J. Photoch. Photobio C **10** (2), 81-95 (2009).
10. Y. Koronyo, D. Biggs, E. Barron, D. S. Boyer, J. A. Pearlman, W. J. Au, and S. Frautschy, JCI insight **2** (16) (2017).
11. V. S. Gota, G. B. Maru, T. G. Soni, T. R. Gandhi, N. Kochar, and M. G. Agarwal, J. Agr. Food Chem. **58** (4), 2095-2099 (2010).
12. E. A. Shirshin, Y. I. Gurfinkel, S. T. Matskeplishvili, M. L. Sasonko, N. P. Omelyanenko, B. P. Yakimov, and M. E. Darvin, J. Biophotonics, e201800066 (2018).
13. E.A. Shirshin, Y.I. Gurfinkel, A.V. Priezzhev, V.V. Fadeev, J. Lademann, M.E. Darvin, Sci. Rep-Uk. **7** (1), 1171 (2017).
14. E. A. Shirshin, O. P. Cherkasova, T. Tikhonova, E. Berlovskaya, A. V. Priezzhev, and V. Fadeev, J. Biomed. Opt. **20** (5), 051033 (2015).
15. E. A. Shirshin, B. P. Yakimov, S. A. Rodionov, N. P. Omelyanenko, A. V. Priezzhev, V. V. Fadeev, and M. E. Darvin, Laser Phys. Lett. **15** (7), 075604 (2018).
16. A. Barik, K. I. Priyadarsini, and H. Mohan, Photochem. Photobiol. **77** (6), 597-603 (2003).
17. A. C. P. Reddy, E. Sudharshan, A. A. Rao, and B. R. Lokesh, Lipids **34** (10), 1025-1029 (1999).

18. A. Barik, B. Mishra, A. Kunwar, and K. I. Priyadarsini, Chem. Phys. Lett. **436** (1-3), 239-243 (2007).
19. J. S. Mandeville, E. Froehlich, and H. A. Tajmir-Riahi, J. Pharmaceut Biomed. **49** (2), 468-474 (2009).
20. J. Schleusener, J. Lademann, and M.E. Darvin, J. Biomed. Opt. **22** (9), 091503 (2017).
21. V. V. Tuchin and V. Tuchin (2007), *Tissue optics: light scattering methods and instruments for medical diagnosis*, 2nd ed. (SPIE Press, Bellingham, WA, 2007)
22. Sdobnov, A. Y., Darvin, M. E., Genina, E. A., Bashkatov, A. N., Lademann, J., and Tuchin, V. V., Spectrochim. Acta. A. **197**, 216-229 (2018).
23. J. T. Alander, I. Kaartinen, A. Laakso, T. Pätilä, T. Spillmann, V. V. Tuchin, and P. Välisuo, Int. J. Biomed. Imaging **7** (2012).
24. D. Magde, R. Wong, and P. G. Seybold, Photochem. Photobiol. **75** (4), 327-334 (2002).