In vivo optical clearing of human skin under the effect of aqueous solutions of some monosaccharides

K.V. Berezin*[a], K.N. Dvoretsky[b], M.L. Chernavina[a], V.V. Nechaev[c], A.M. Lichter[d], V.V. Smirnov[d], E.M. Antonova[e], O.N. Grechukhina[f], M.V. Kartashov[f], V.V. Tuchin[a,g,h]

[a] Saratov State University, Saratov, Russian Federation; [b] Saratov State Medical University, Saratov, Russian Federation; [c]Saratov State Technical University, Saratov, Russian Federation; [d]Astrakhan State University, Astrakhan, Russian Federation; [e]Astrakhan State Medical University, Astrakhan, Russian Federation; [f]Caspian University of Maritime and River Transport, Astrakhan, Russian Federation; [g]Institute of Precision Mechanics and Control, Russian Academy of Sciences, Saratov, Russian Federation; [h]National Research Tomsk State University, Tomsk, Russian Federation
* berezinkv@yandex.ru

ABSTRACT

The results of in vivo optical immersion clearing of human skin under the effect of aqueous solutions of some immersion agents (monosaccharides of ribose, glucose and fructose, as well as glycerol, a triatomic alcohol) were obtained with the use of the OCT method. Values of average velocity of scattering coefficient change, obtained through an averaged A-scan of the OCT signal in the region of derma with the depth of 350 to 700 µm, were determined to evaluate the optical clearing efficiency. The velocity of scattering coefficient change and the optical clearing potential value appeared to be well correlated. The complex molecular modeling of a number of immersion clearing agents with a mimetic peptide of collagen $(GPH)_3$, carried out with the use of the methods of classical molecular dynamics and quantum chemistry, allowed to identify correlations between the optical clearing efficiency and such a property as the energy of intermolecular interaction of clearing agents with a collagen peptide fragment.

Keywords: Molecular modeling, immersion optical clearing of biological tissues, collagen, molecular dynamics, OCT

1. INTRODUCTION

The application of modern methods of photomedicine and biomedical optics for diagnosis and therapy of diseases entails difficulties arising because of strong scattering in visible and near-infrared regions inherent to skin and many other biological tissues. This scattering happens due to inhomogeneity of refractive indices at borders of different macromolecular structures, basically on collagen fibers that are primarily responsible for light scattering of skin.[1] These difficulties can be overcome with injection of biocompatible molecular agents into the tissue, which to some extent facilitates to its optical clearing.[2–5] A lot of papers[6–12] are dedicated to in vivo and in vitro experimental studies on clearing of different biological tissues, which proves the urgency of the issue. In the paper,[13] the synergistic effect of an immersion agent (PEG 400), two penetration enhancers (triazine and 1,2-propanediol) and physical massage on the efficiency of in vivo optical clearing of rat skin was evaluated with the use of OCT methods. The paper[14] presents a mathematical model of light propagation in biological tissues. The paper[15] describes an OCT-based development of a technology for noninvasive identification of local molecular diffusion of immersion agents. The paper[16] describes the model diabetes mellitus effect on optical clearing of laboratory mouse skin. The mechanism of skin optical clearing with glycerin as a clearing agent was studied in[17] through visualization and with the use of the second optical harmonic (SHG-imaging). Nevertheless, the mechanisms of optical clearing at molecular level have not been identified clearly yet, and there are very few studies[1,18–19] on molecular processes responsible for skin optical clearing. The paper[20] presents the results of the study on clearing agent dehydrating properties and underlines that dehydration is just one of the possible mechanisms which lead to biological tissue clearing. Conducting research in this field provides the understanding of optical clearing processes at molecular level, which, in its turn, will allow to use new efficient clearing agents with tailor-made properties.

Saratov Fall Meeting 2018: Laser Physics, Photonic Technologies, and Molecular Modeling,
edited by Vladimir L. Derbov, Proc. of SPIE Vol. 11066, 110661D · © 2019 SPIE
CCC code: 0277-786X/19/$18 · doi: 10.1117/12.2523317

This paper is sequel to the authors' studies on molecular mechanisms of biological tissue optical clearing. The paper[21–22] describes the study on the interaction of a glycerin immersion agent with a collagen mimetic peptide $((GPH)_9)_3$ and a fragment of microfibril $5((GPH)_{12})_3$ with the use of the method of classical molecular dynamics. The change in geometric parameters of collagen α-chains under different concentrations of a glycerin aqueous solution was analyzed. It was shown that these changes depend nonlinearly on concentration and have a maximum, which correlates well with experimental data on efficiency of human skin optical clearing. A hypothesis was made on a cause of decrease in the skin optical clearing efficiency under high concentrations of an immersion agent.

The papers[23–25] are dedicated to the studies on interaction of six biotissue-clearing immersion agents (1,2 and 1,3-propanediol, ethylene glycol, glycerol, xylitol and sorbitol) with a collagen mimetic peptide $(GPH)^3$ with the use of the methods of classical molecular dynamics, molecular docking and quantum chemistry (PM6 and DFT/B3LYP). Correlations between the optical clearing potential and such parameters of intermolecular interaction as the time of a hydrogen-bonding state of agents, relative probability of double hydrogen bond formation and complex formation energy were identified. The determined correlation allowed to predict a numerical value of the optical clearing potential of a dextrose molecule for rat skin, which is much in agreement with the experimental data. Minor changes in the optical clearing potential during the transition from monosaccharides to disaccharides were explained. A molecular mechanism of the post-diffusion stage of biological tissue optical clearing was suggested.

In the framework of the present paper, we continue studying correlations between the efficiency of biological tissue optical clearing and the energy of forming complexes between clearing immersion agents with a collagen mimetic peptide. A number of monosaccharides – ribose, glucose and fructose – were used as immersion agents.

2. EXPERIMENT METHODS AND RESULTS

Aqueous solutions (60%) of the following immersion agents were used to study optical clearing of skin: monosaccharides (ribose, glucose and fructose) and, for comparison, glycerol, a triatomic alcohol. The optical coherence tomography (OCT) was used to evaluate the influence of clearing properties of immersion liquids on skin during in vivo experiments. Visualization was carried out with the use of Thorlabs OCP930SR (Thorlabs, USA), an optical coherence tomograph, with the following parameters: radiation wave central length of 930±5 nm, axial and lateral resolution of 6.2 and 9.6 μm respectively (in the air), scan region length of 2 mm.

The measurements were carried out on a skin area of the back of a forearm. The scans were recorded before the exposure with immersion agents, then at 1-minute intervals during 40 minutes of exposure. The measurements involved four volunteers, and a total of five experiments was carried out for each immersion agent.

The attenuation coefficient μ_t[26] was evaluated by the OCT scans skew on the basis of the single scattering model[27–28]. According to this model, the registered OCT signal power R(z) is proportional to $\exp(-\mu_t z)$.[29] As the absorption factor μ_a is far less that the scattering coefficient μ_s[1] in the spectral range under study, the attenuation coefficient $\mu_t = \mu_s + \mu_s$ may be considered approximately equal to the scattering coefficient, so the value R(z) may be approximated by the expression $R(z) = A\exp(-\mu_s z) + B$, where A is the proportionality factor equal to $P_0 \cdot a(z)$, P_0 is the optical power of the beam falling on the biological tissue surface, a(z) is determined by the biotissue local ability to disperse light backwards, which depends on a local variation of the refraction index, and B is the background signal. The selection of coefficients in the above expression for approximation of the experimental curve allows to evaluate a depth-averaged (efficient) light scattering coefficient by the tissue sample.

Figure 1 presents analyzed areas of the OCT image, as well as an averaged A-scan of the OCT signal of the human skin dermal layer in vivo (5 minutes after applying 60% ribose solution on the surface) and an approximating curve plotted with the use of the single scattering model. OCT signals were averaged by the A-scan over the whole area of scanning. The scattering coefficient values were determined in the region of the averaged A-scan at the depth of 350 to 700 μm.

Within the framework of the present paper, values of the light scattering coefficient, obtained with the use of the averaged A-scan in the derma region with the depth of 350 to 700 μm were determined to evaluate the efficiency of in vivo optical clearing of human skin. Figure 2 presents temporal dependences of the obtained scattering coefficients under exposure to 60% aqueous solutions of three monosaccharides and one trialcohol glycerol.

Values of the module of average velocity of the scattering coefficient change at the time segment from 5 to 35 minutes were used for numerical expression of the skin optical clearing efficiency. These velocities are presented in the graph as skew value in regression line equations.

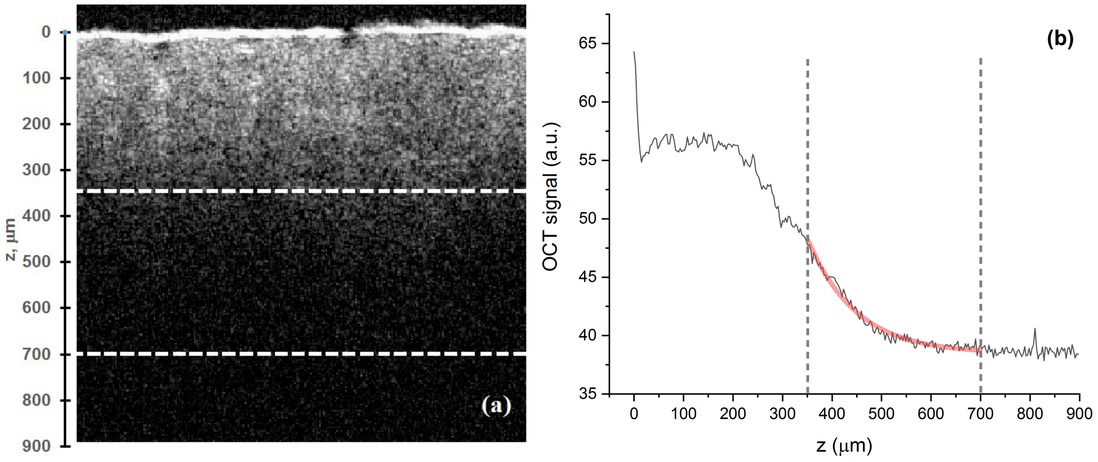

Figure 1. Measurements of the scattering coefficient µs in the derma region with the depth of 350 to 700 µm on the basis of the analysis of depth distribution of the averaged OCT signal with the use of the single scattering model; (a) is the in vivo fragment of the B-scan of skin which was used to average the OCT signal, (b) is the depth distribution of the averaged OCT signal (thin curve) and the approximation result according to the single scattering model (thick curve). The dashed lines are borders of the regions where the value µs was determined.

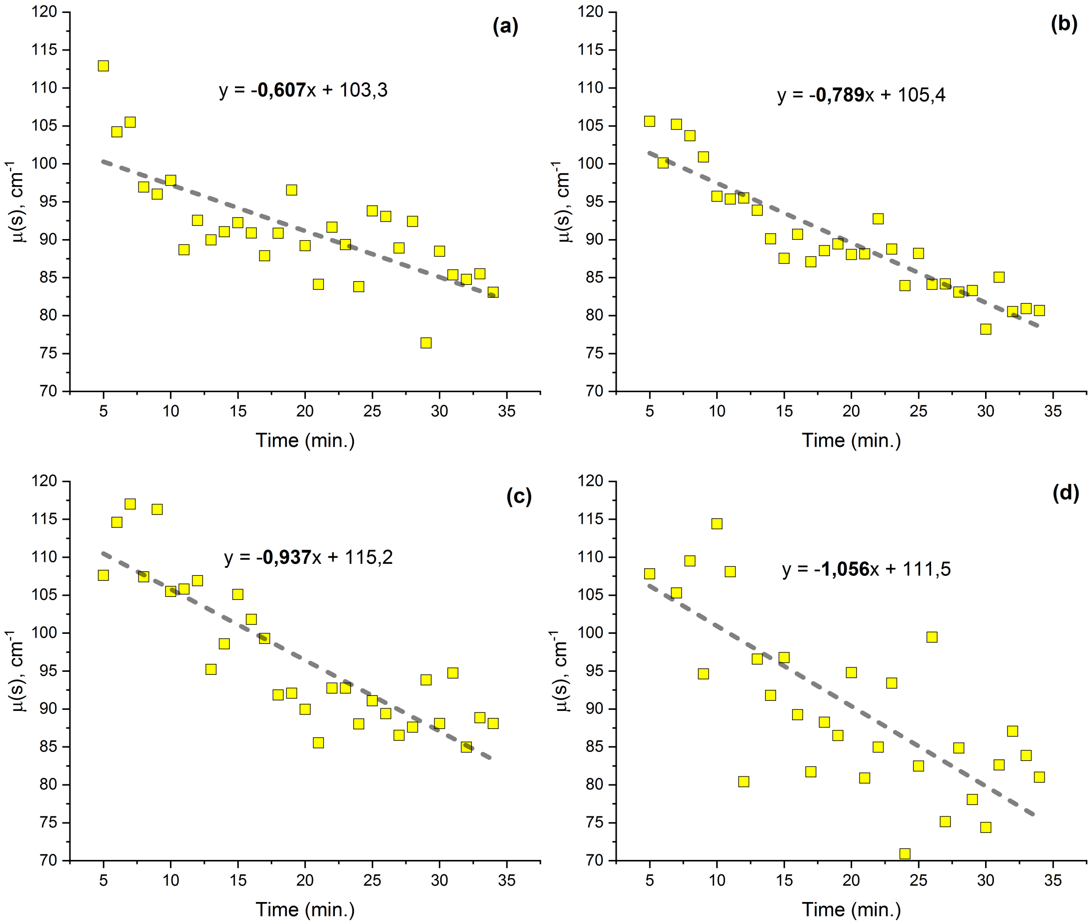

Figure 2. Dependence of the scattering coefficient value µs in the derma region with the depth of 350 to 700 µm, based on the analysis of the depth distribution of the averaged OCT signal with the use of the single scattering model, on the effect of immersion agents: (a) – glycerol; (b) – ribose; (c) – glucose and (d) – fructose.

3. MOLECULAR MODELING METHODS AND RESULTS

As is the previous papers,[21–24] a mimetic peptide of collagen (GPH)^3,[30] forming the basis of a great part of regular domains of human collagen, was used as a molecular model of collagen. Such relatively small synthetic peptides are often used for collagen molecular modeling. A peptide 3D model was built according to the Protein Data Bank (PDB) data with further addition of hydrogen atoms and optimization of the structure by the molecular mechanics method.[31] A number of monosaccharides (ribose, glucose and fructose) were considered as immersion clearing agents. The molecular modeling of interaction of clearing agents with collagen was carried out in two stages.

At the first stage, the method DFT/B3LYP/6–311+G(d,p)[32–33] and the programme Gaussian[34] were used to identify and calculate all the lowest energy conformers of the considered monosaccharides in their isolated state. The calculated geometric parameters and values of the Mulliken atomic charges were further used in modeling of these systems within the framework of classical molecular dynamics. Vibrational transition wavenumbers were also calculated and appeared to be positive, which gives another evidence of molecular systems being at the local minimum. Spatial configurations of the lowest energy conformers of some clearing agents are shown in (Fig.3a) and (Fig.3b).

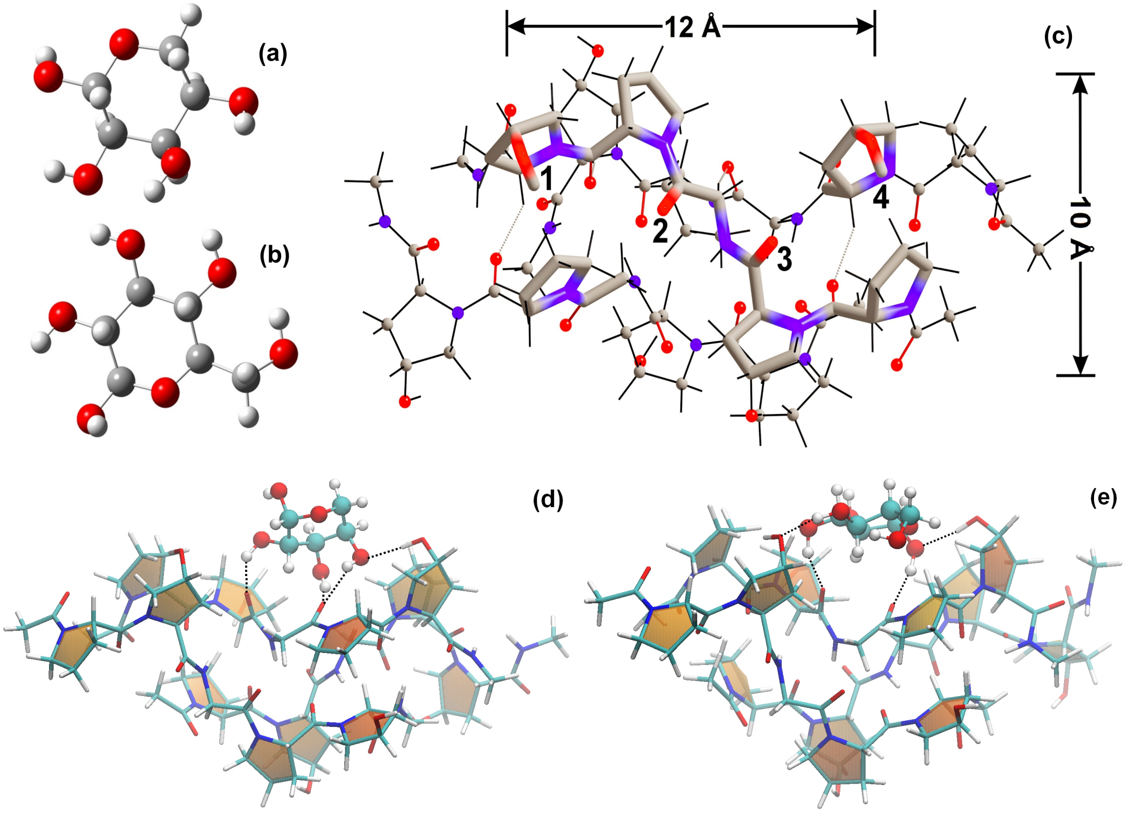

Figure 3. Spatial configurations: (a, b) – the lowest energy conformers of some clearing agents (ribose and glucose); (c) – fragment of the mimetic peptide $((GPH)_3)_2$ optimized within the semi-empirical method PM6 (figures stand for molecular groups participating in formation of hydrogen bonds with clearing agents); (d, e) – hydrogen-bonding complexes, formed by the collagen fragment $((GPH)_3)_2$ and the above immersion clearing agents. Hydrogen bonds are shown with dash lines.

At the second stage, a minimum fragment of the mimetic peptide, preserving the regular structure $((GPH)_3)_2$, consisting of 231 atoms and having a structure that was preliminary optimized within the semi-empirical method PM6, was used to evaluate the energy of intermolecular interaction of the chosen clearing agents with collagen [35]. The obtained spatial structure is shown in (Fig. 3c). As is seen from (Fig. 3c), an entry molecular pocket is a peptide region with an approximate size of 10x12 Å that has four functional groups available for intermolecular bonding: two carbonyl groups (one at the glycine residue (2) and the other at the hydroxyproline residue (3) of the same α-chain) and two alcohol groups (1 and 4) at the hydroxyproline residues of different α-chains. This optimized structure of the collagen model was used to carry out molecular docking with clearing agents within the program AutoDockVina.[36]

When the molecular docking was carried out, the first five most suitable configurations were selected for each interacting system, and then they were optimized with the semi-empirical method PM6. Then the total electronic energy of the complexes was calculated with the method DFT/B3LYP/6-31G(d) and through the single SCF procedure. A similar procedure was used to obtain values of the total electronic energy of the clearing agents and the peptide fragment. The intermolecular interaction energy was calculated as a difference between the complex total energies and the sum of energies of its specific components. The largest values of intermolecular interaction energies, corresponding to the most probable complex structures, were chosen to identify correlation with the optical clearing efficiency. Figure 3 (d, e) shows a PM6 obtained spatial structure of hydrogen-bonding complexes, formed by the collagen fragments $((GPH)_3)_2$ and some clearing agents.

4. DISCUSSION AND FINDINGS

To make a discussion on the obtained results more convenient, the qualitative parameters of intermolecular interactions (the values of length of classical hydrogen bonds, formed according to calculations between active groups of the collagen molecular pocket and hydroxyl groups of clearing agents, and calculated values of intermolecular interaction energies), as well as values of the module of average velocity of scattering coefficient change, experimentally obtained with the use of OCT, are presented in Table 1.

Table 1. Lengths of hydrogen bonds (in angstroms), energies of intermolecular interactions (in kJ/mol) between the fragments of collagen $(GPH)_3$ and different clearing agents, calculated with the use of the method PM6/B3LYP/6-31G(d), as well as experimental values of optical clearing velocity.

Type of agent	Hydrogen bond lengths	ΔE	Efficiency of skin optical clearing
glycerol	1.74; 1.91; 1.92; 1.93; 2.44	-42.8	0.607
ribose	1.84; 1.90; 1.91; 1.95	-80.9	0.789
glucose	1.68; 1.71; 1.84; 1.94	-94.5	0.937
fructose	1.82; 1.84; 1.90; 1.96; 2.23	-89.2	1.056

For example, as can be seen from Table 1, a molecule of trialcohol glycerol forms four relatively strong hydrogen bonds and one weaker bond with all active groups of the entry pocket; however, the molecule length is insufficient to make all the hydrogen bonds efficient. The transition from trialcohol glycerol to glucose monosaccharide demonstrates a significance increase in the skin optical clearing efficiency. It can be explained by the fact that, according to the calculation, glucose forms stronger hydrogen bonds with collagen than glycerol does. Table 1 shows that, despite fewer hydrogen bonds, their length is notably shorter, which is a determining factor for bonding energy. As is seen from (Fig.3), stronger hydrogen bonds with carbonyl groups are formed both due to a compact ring structure of glucose that allows to low into a collagen molecular pocket and due to good mutual disposition of interacting groups.

Various parameters are used by different authors to evaluate the degree (efficiency) of optical clearing with immersion agents. For example, in[1] they use the optical clearing potential, introduced as a slope of the dependence of reduced scattering coefficient after 45 minutes of immersion agent effect on its concentration, expressed in moles, i.e. for its determination it is necessary to study the effect of immersion agents with different initial concentrations.

In this paper, values of the module of average velocity of scattering coefficient change under effect of the solution of a moderate-concentration immersion agent were used for numerical expression of the skin optical clearing efficiency.

As is seen in Figure 4, these two parameters correlate well with each other. It allows us to use the value of clearing velocity in further research as a way to evaluate the efficiency of optical clearing with immersion agents.

Figure 4 also shows that the energies of interaction between a collagen peptide molecule and molecules of different clearing agents, calculated with the use of the method PM6/B3LYP/6-31G (spirit values are taken from [22]), correlate well both with the value of the potential of rat and human skin optical clearing [1] and with the experimentally obtained values of velocity of human skin scattering coefficient change.

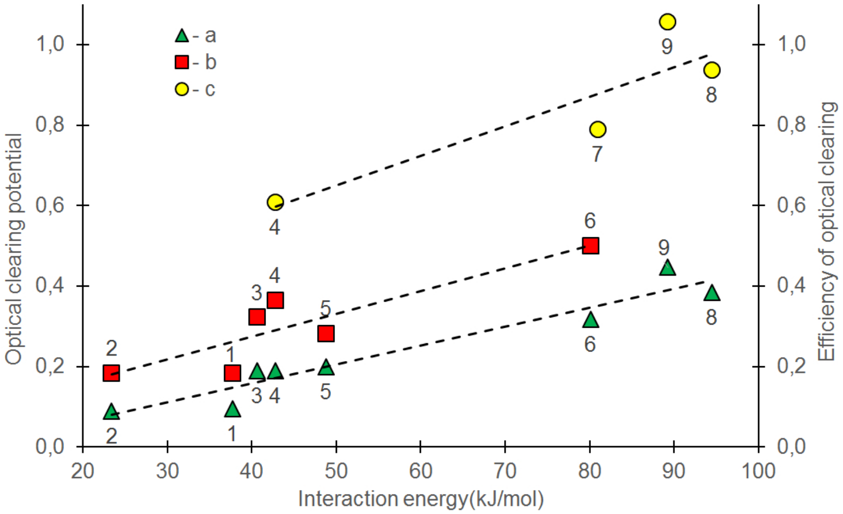

Figure 4. Dependence of the value of the optical clearing potential (left axis) of rat skin (a) and human skin (b) [1], as well as values of the efficiency of optical clearing (right axis) of human skin (c) on the energy of interaction between a collagen peptide molecule and molecules of clearing agents. Immersion agents are indicated with numbers: 1 - ethylene glycol; 2 – 1,2-propanediol; 3 - 1,3- propanediol; 4 – glycerol; 5 – xylitol; 6 – sorbitol; 7 – ribose; 8 – glucose and 9 – fructose.

It is therefore possible to speak of fundamental importance of a post-diffusion stage, where collagen interacts with clearing agents and influences optical clearing of biological tissues. The study results allow to suggest that during the process of this interaction, a partial substitution of collagen-related water occurs. It leads to disturbance in a binding net of hydrogen bonds and, consequently, to a reversible process of collagen fibril dissolution, which, in its turn, decreases their value of deflection index and equalizes it to the intercollagen medium. The higher collagen affinity of a clearing agent, the more effective the process is.

The next essential step to increase the interaction efficiency is choosing a molecular agent with specific structural characteristics that will allow it to interact with two or more molecular pockets of collagen at the same time. Such an effective clearing agent can be a molecular system of polymeric type, consisting of, for example, hexatomic monosaccharides, bonded by a moveable carbon-oxygen chain with the length that will allow saturated sugar rings to reach the areas of molecular pockets of collagen and interact with them through their hydroxyl groups. It should be noted that a significant increase in the size of molecules used as clearing agents will lead to the substance viscosity growth and, as a consequence, to reduction of its diffusion coefficient in the biological tissue, as well as to increment of time necessary for its washout from the tissues.

5. ACKNOWLEDGMENTS

The authors express their gratitude to E.A. Genina, A.N. Bashkatov and D.K. Tuchina for her help in conducting the experiments.

The research was partially supported by the grants of the Russian Foundation for Basic Research No. 18-52-16025a and 18-07-01228a, and also financially supported within the framework of the state tasks to higher educational institutions and research organizations in the sphere by scientific activities from the Ministry of Education and Science No. 3.9128.2017/БЧ.

REFERENCES

[1] Hirshburg J.M., [Chemical agent induced reduction of skin light scattering: doctoral dissertation] Texas A&M University (2009).
[2] Tuchin V. V. (ed.), [Handbook of optical sensing of glucose in biological fluids and tissues] Taylor & Francis Group LLC, CRC Press (2009).

[3] Tuchin V. V., [Optical clearing of tissues and blood] PM 154, SPIE Press, Bellingham, WA (2006).
[4] Zhu D., Larin K.V., Luo Q. and Tuchin V.V., "Recent progress in tissue optical clearing," Laser Photonics Rev. 7, 732-757 (2013).
[5] Genina E.A., Bashkatov A.N., Sinichkin Yu.P., Yanina I.Yu. and Tuchin V.V., "Optical clearing of biological tissues: prospects of application in medical diagnostics and phototherapy [Review]," J. Biomed. Photonics & Eng., 1(1), 22-58 (2015).
[6] Genina E.A., Bashkatov A.N., Kochubei V.I. and Tuchin V.V., "Optical clearing of human dura mater," Optics and Spectroscopy 98(3), 470-476 (2005).
[7] Genina E.A., Bashkatov A.N., Sinichkin Yu.P. and Tuchin V.V., "Optical clearing of the eye sclera in vivo caused by glucose," Quantum Electronics 36(12), 1119-1124 (2006).
[8] Bashkatov A.N., Korolevich A.N., Tuchin V.V., Sinichkin Yu.P., Genina E.A., Stolnitz M.M., Dubina N.S., Vecherinski S.I. and Belsley M.S., "In vivo investigation of human skin optical clearing and blood microcirculation under the action of glucose solution," Asian J. Physics 15, 1-14 (2006).
[9] Genina E.A., Bashkatov A.N. and Tuchin V.V., "Optical clearing of cranial bone," Adv. Optical Technologies 2008, Article ID 267867 (2008).
[10] Bashkatov A.N., Genina E.A., Tuchin V.V. and Altshuler G.B., "Skin optical clearing for improvement of laser tattoo removal," Laser Physics 19(6), 1312-1322 (2009).
[11] Wen X., Tuchin V.V., Luo Q. and Zhu D., "Controling the scattering of intralipid by using optical clearing agents," Physics in Medicine and Biology 54(22), 6917-6930 (2009).
[12] Sudheendran N., Mohamed M., Ghosn M.G., Tuchin V.V. and Larin K.V., "Assessment of tissue optical clearing as a function of glucose concentration using optical coherence tomography," J. Innovative Optical Health Sciences 3(3), 169-176 (2010).
[13] Wen X., Jacques S., Tuchin V. and Zhu D., "Enhanced optical clearing of skin in vivo and optical coherence tomography in-depth imaging," J. Biomed. Opt. 17(6), 066022 (2012).
[14] Simonenko G.V., Kirillova E.S. and Tuchin V.V., "Mathematical model for describing of kinetics of tissue optical clearing," Optical Memory & Neural Networks 18(2), 129-133 (2009).
[15] Larin K.V. and Tuchin V.V., "Functional imaging and assessment of the glucose diffusion rate in epithelial tissues in optical coherence tomography," Quantum Electronics 38(6), 551-556 (2008).
[16] Tuchina D.K., Shi R., Bashkatov A.N., Genina E.A., Zhu D., Luo Q. and Tuchin V.V., "Ex vivo optical measurements of glucose diffusion kinetics in native and diabetic mouse skin," Journal of Biophotonics 8(4), 332-346 (2015).
[17] Wen X., Mao Z., Han Z., Tuchin V.V. and Zhu D., "In vivo skin optical clearing by glycerol solutions: mechanism," J. Biophoton. 3(1-2), 44-52 (2010).
[18] Hirshburg J.M., Ravikumar K.M., Hwang W. and Yeh A.T., "Molecular basis for optical clearing of collagenous tissues," J. Biomed. Opt. 15, 055002 (2010).
[19] Feng W., Shi R., Ma N., Tuchina D.K., Tuchin V.V. and Zhu D., "Skin optical clearing potential of disaccharides," J. Biomed. Opt. 21, 081207 (2016).
[20] Yu T., Wen X., Tuchin V.V., Luo Q. and Zhu D., "Quantitative analysis of dehydration in porcine skin for assessing mechanism of optical clearing," J. Biomed. Opt. 16, 095002 (2011).
[21] Dvoretsky K.N., Berezin K.V., Chernavina M.L., Likhter A.M., Shagautdinova I.T., Antonova E.M., Rybakov A.V., Grechukhina O.N. and Tuchin V.V., "Molecular modeling of the process of reversible dissolution of the collagen protein under the action of tissue-clearing agents," Proc. SPIE 10716, 1071624 (2018).
[22] Dvoretsky K.N, Berezin K.V., Chernavina M.L., Likhter A.M., Shagautdinova I.T., Antonova E.M., Grechukhina O.N. and Tuchin V.V., "Molecular Modeling of the Post-Diffusion Stage of Surface Bio-Tissue Layers Immersion Optical Clearing," Journal of Surface Investigation 12(5), 961-967 (2018).
[23] Berezin K.V., Dvoretski K.N., Chernavina M.L., Likhter A.M., Smirnov V.V., Shagautdinova I.T., Antonova E.M., Stepanovich E.Yu., Dzhalmuhambetova E.A. and Tuchin V.V., "Molecular modeling of immersion optical clearing of biological tissues," J. Mol. Model. 24, 45 (2018).
[24] Berezin K.V., Dvoretskiy K.N., Chernavina M.L., Nechaev V.V., Likhter A.M., Shagautdinova I.T., Stepanovich E.Yu., Grechukhina O.N. and Tuchin V.V., "Studying the mechanism of tissue optical clearing using the method of molecular dynamics, " Proc. SPIE 10336, 103360J (2017).
[25] Bashkatov A.N., Berezin K.V., Dvoretskiy K.N., Chernavina M.L., Genina E.A., Genin V.D., Kochubey V.I., Lazareva E.N., Pravdin A.B., Shvachkina M.E., Timoshina P.A., Tuchina D.K., Yakovlev D.D., Yakovlev

D.A., Yanina I.Yu., Zhernovaya O.S. and Tuchin V.V., "Measurement of tissue optical properties in the context of tissue optical clearing," J. Biomed. Opt. 23(9), 091416 (2018).

[26] Lee P., Gao W. and Zhang X., "Performance of single-scattering model versus multiple-scattering model in the determination of optical properties of biological tissue with optical coherence tomography," Appl. Opt. 49(18), 3538-3544 (2010).

[27] Faber D.J., van der Meer F.J., Aalders M.C.G. and van Leeuwen T.G., "Quantitative measurement of attenuation coefficients of weakly scattering media using optical coherence tomography," Opt. Express 12(19), 4353-4365 (2004).

[28] Genina E., Bashkatov A., Kolesnikova E., Basko M., Terentyuk G. and Tuchin V., "Optical coherence tomography monitoring of enhanced skin optical clearing in rats in vivo," J. Biomed. Opt. 19(2), 021109 (2013).

[29] Wang R.K. and Tuchin V.V. [Handbook of Coherent-Domain Optical Methods. Biomedical Diagnostics, Environmental Monitoring, and Material Science.], Springer vol. 2., 665 (2013).

[30] Okuyama K., Miyama K., Mizuno K. and Bachinger H.P., "Crystal structure of (Gly-Pro-Hyp)9: implications for the collagen molecular model," Biopolymers 97(8), 607-616 (2012).

[31] Cornell W.D., Cieplak P., Bayly C.I., Gould I.R., Merz K.M.Jr., Ferguson D.M., Spellmeyer D.C., Fox T., Caldwell J.W. and Kollman P.A., "A second generation force field for the simulation of proteins, nucleic acids, and organic molecules," J. Am. Chem. Soc. 117(19), 5179-5197 (1995).

[32] Becke A.D., "Density-functional thermochemistry. III. The role of exact exchange," J. Chem. Phys. 98(7), 5648-5652 (1993).

[33] Lee C., Yangand W. and Parr R.G., "Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density," Phys. Rev., 37B(2), 785-789 (1988).

[34] Frisch M.J., Trucks G.W., Schlegel H.B. et al., Gaussian09, Revision A.02. Pittsburgh PA: Gaussian, Inc., (2009).

[35] van der Spoel D., Lindahl E., Hess B., Groenhof G., Mark E.A. and Berendsen H.J.C., "GROMACS: Fast, flexible and free," J. Comput. Chem., 26(16), 1701-1718 (2005).

[36] Duan Y., Wu C., Chowdhury S., Lee M.C., Xiong G., Zhang W., Yang R., Cieplak P., Luo R., Lee T., Caldwell J., Wang J. and Kollman P., "A point-charge force field for molecular mechanics simulations of proteins based on condensed-phase quantum mechanical calculations," J. Comput. Chem., 24(16), 1999-2012 (2003).