Journal of Photochemistry and Photobiology B: Biology 103 (2011) 230–233

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Penetration of laser light through red blood cell ghosts

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ARTICLE INFO

Article history:
Received 14 December 2010
Received in revised form 9 March 2011
Accepted 21 March 2011
Available online 29 March 2011

Keywords:
Laser light penetration
Red blood cell
Hemolysis
Hemoglobin release
Phototherapy

ABSTRACT

Hemoglobin is the main absorber of visible light in blood and blood-perfused tissues. However, hemoglobin is released from a red blood cell (RBC) during hemolysis. Hemolysis may be caused by a large number of medical conditions, including photodynamic therapy (PDT) and this subsequently can affect passage of light through the treated biological structures. The purpose of the present study was to determine the penetration of a laser beam through a suspension of hemoglobin-free human red blood cells (RBCs) – ghosts. Although hemoglobin has been efficiently removed from the samples used in our experiments, our measurements show that the samples still effectively attenuate the radiant power of penetrating laser light. We established penetration depths of 12.6 mm and 15.4 mm for two different laser light wavelengths, 532 nm and 630 nm, respectively. The penetration depth of laser light was about one order of magnitude higher for hemoglobin-free RBC ghosts as compared to intact RBCs [8,10,12]. These results can be important in case of phototherapy or biostimulation, since all photons that penetrate in a biological object may interact with it and evoke biological response.

1. Introduction

The propagation of laser light in tissues is a question of growing importance in many diagnostic and therapeutic applications in today's photo-medicine. Lasers, suitable light sources for photomedical applications, provide highly monochromatic, temporally and spatially coherent light with high stability of radiation. Distribution of laser light in a tissue may considerably differ for various tissues depending on their structure and composition [1–3]. From the point of view of optics, a biological tissue is an inhomogeneous medium containing absorbing inhomogeneities and fluctuations of refractive index across cell membranes, organelles, etc. [4]. All blood-perfused tissues contain red blood cells (RBCs), also known as erythrocytes, which are mainly composed of hemoglobin, water and membrane components. In mammals, erythrocytes have no nuclei and also lose all other cellular organelles such as mitochondria, Golgi apparatus and endoplasmic reticulum. Thus they contain no internal membrane structures and provide a good opportunity to apply various mathematical models [5–7]. To-date, optical parameters describing light absorption and scattering on a single erythrocyte and a mass of erythrocytes were experimentally determined [7–14]. Spectral properties of hemoglobin have been thoroughly investigated as well [15–17]. Due to the heme prosthetic group, hemoglobin is the main absorber of visible light in blood and blood-perfused tissues. However, hemoglobin is released from RBC during hemolysis both *in vivo* and *in vitro*. *In vitro* hemolysis can be caused by improper handling during blood collection, or by bacterial action. *In vivo* hemolysis can be caused by a large number of medical conditions, including many Gram-positive bacteria, some parasites, some genetic or autoimmune disorders. Hemolysis can also occur in some therapies, particularly in photodynamic therapy (PDT) or photodynamic sterilization of blood (PDS) [18]. This can subsequently affect propagation of light through the treated biological structures. It is therefore important, in particular with photodynamic treatments, to study the effect of hemoglobin removal from the RBCs on passage of light through a sample. This study is focused on penetration of laser light through a suspension of hemoglobin-free human red blood cells (ghosts).

2. Materials and methods

Fresh human blood was collected under the guidelines of the Helsinki Declaration for human research at Slovak Medical University, Bratislava. Venous blood from healthy adult donors, aged 20–63 years, was drawn into syringes filled with heparin as an anticoagulant.

Erythrocytes were obtained by centrifugation at 916 g for 10 min at temperature 4 °C. Excess plasma and platelets were removed and erythrocytes were washed three times with ice cold phosphate buffered saline (PBS) (10 mmol/L phosphate buffer, 150 mmol/L NaCl, pH 7.4). To obtain a suspension of hemoglobin-free RBC ghosts in 5 mmol/L Tris (pH 7.4) buffer, approximately one volume of erythrocytes was hemolysed in five volumes of 20 mmol/L Tris buffer (pH 7.4) and centrifuged at 19 621 g for 10 minutes at temperature 4 °C, followed by double washing with

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doi:10.1016/j.jphotobiol.2011.03.015

20 mmol/L, 10 mmol/L and 5 mmol/L Tris buffer, respectively [19]. All centrifugations were carried out at 4 °C using the Universal 320R centrifuge (Hettich Zentrifugen, Germany). As in freshly prepared RBC ghosts (within 1 hour), we observed no ghosts fragments, in accordance with [19], light penetration measurements were carried out within 30 minutes of the preparation of the ghosts.

Photoirradiation of ghosts was carried out with two different continuous wave lasers. We used the second harmonic of an NdYAG laser (532 nm, 30 mW) from Raise Electronic, Taiwan, and Laser Diode Module (630 nm, 30 mW) from Kvant, Slovakia. Laser light penetration through samples was determined by direct measurements of spectral radiant power transmitted through the sample along the centerline of the incident laser beam. The spectral radiant power was measured by a LaserCheck detector (Coherent, Germany). The registration time was 2 s. The values of radiant power were corrected for transmittance of the glass container where the blood derivatives were placed. Absorption spectra of RBC ghosts were recorded by a PharmSpec UV-1700 spectrophotometer (Shimadzu, Japan).

Prior to each measurement, the suspension of ghosts was stirred on a minishaker (MS2 Minishaker, USA). All measurements were carried out at laboratory temperature (22 °C ± 1 °C).

3. Results and discussion

The studied samples were first checked for residues of hemoglobin. For this purpose, we compared absorption spectra of RBCs with those of RBC ghosts (Fig. 1a). The spectrum of intact RBCs possesses the absorption bands characteristic of hemoglobin – the Soret band at 415 nm, and Q bands at 544 nm and 578 nm [20]. However, in the same absorption region the spectrum of RBC ghosts shows a negligible absorbance with no significant peaks visible (Fig. 1a). Only a large zoom reveals a minor absorption peak at 415 nm, attributable to the hemoglobin Soret band. However, no hemoglobin absorption side bands were observed between 500 and 650 nm. We can conclude that absorbance of RBC ghosts at the monitored wavelengths (532 and 630 nm) is negligible and hemoglobin has been sufficiently removed from the ghosts. This was reached by hypotonic hemolysis and a repeated washing/centrifugation procedure. It is well known that normal erythrocytes lyze when exposed to a hypotonic solution, and ruptured RBCs release their contents (inclusive hemoglobin) into the medium [21]. Hemoglobin was removed from the medium by repeated (at least six times) washing and centrifugation, which improved the removal of hemoglobin from the sample (Fig. 1b) compared to the previous study [19], where the sample was washed four times. Although hemoglobin is not a structural element of biomembranes, several authors report cases when a fraction of hemoglobin remains bound to RBC membranes. This includes, in particular, some pathological conditions like sickle anemia [22], the presence of external substances (dextran, ethylene glycol, albumin, or hemoglobin itself) [21], and ghosts prepared at moderate osmolarities or at acidic pH [19]. None of these conditions applies to our study.

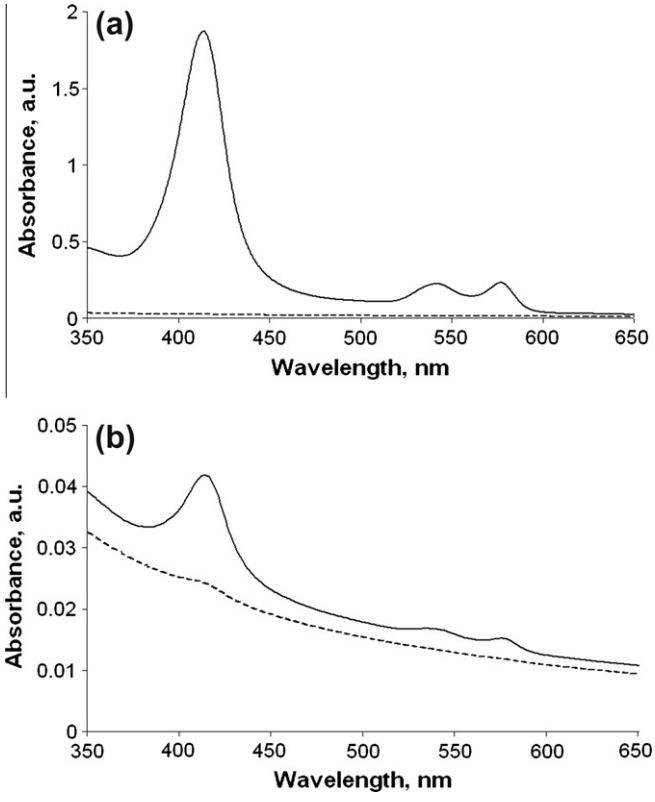

Fig. 1. Comparison of absorption spectra of intact RBCs (solid line) and RBC ghosts (dashed line) (a); zoomed spectrum of RBC ghosts after 6 (solid line) and 4 (dashed line) washings with a hypotonic solution (b).

Penetration of laser light through suspensions of red blood cell ghosts was studied by direct measurements of radiant power for two different incident light wavelengths, 532 nm and 630 nm. Our data showed that on the central axis of the irradiation beam the spectral radiant power decreased exponentially with the depth in the sample for both wavelengths used (Fig. 2). The observed exponential attenuation of radiant power is in accordance with the so-called modified Beer–Lambert law for turbid media, such as biological tissues [23,24]. This law can be expressed as

$$P(d) = P_0 exp\{-\mu.d\} \quad (1)$$

where P_0 is the spectral radiant power for d approaching zero from the side of the sample; μ is the attenuation coefficient, depending on absorption and scattering characteristics of the sample. The inverse of the attenuation coefficient μ is the optical penetration depth δ, which is the depth at which the spectral radiant power in a medium is reduced by a factor of 1/e [23,25].

$$\delta = 1/\mu \quad (2)$$

Application of Eq. (1) to the experimental spectral radiant power data for penetration of laser light through a suspension of RBC ghosts gives fits with relatively high values of coefficient of determination, R^2, and low values of the non-linear chi-square, χ^2. It is therefore possible to estimate the optical parameters μ, δ, P_0 (Table 1) in a simple manner and from direct measurements.

It is well known that red blood cells (RBCs) are largely responsible for the optical behavior of blood. They prevail in blood and contain a strongly absorbing metalloprotein, hemoglobin, with a refractive index that influences the absorption and scattering properties of RBCs in the VIS wavelength range [26]. Hemoglobin has been efficiently removed from the RBC ghosts samples used in our experiments; however, our measurements show that the samples still effectively attenuate the radiant power of penetrating laser light (Fig. 2). Due to the low hemoglobin content of the sample, the contribution of sample absorption to light attenuation at 532 nm and 630 nm is negligible, but there may be a substantial contribution of scattering and diffused reflection here. The ghosts carry the complex three-layer structure of RBC membranes: the glycocalyx on the exterior, which is rich in carbohydrates; the lipid bilayer, which contains, besides its lipidic main constituents, many transmembrane proteins; and the membrane skeleton, a structural network of proteins located on the inner surface of the lipid bilayer. In human erythrocytes, half of the membrane mass are