Confocal Raman microspectroscopy for evaluation of optical clearing efficiency of the skin *ex vivo*

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Abstract

Optical clearing (OC) of biological tissues is a promising technology for a wide spread use in medical practice to increase the screening depth, spatial resolution and contrast of the resulting images/spectra. Nevertheless, despite the significant OC effect, some biocompatible optical clearing agents (OCAs) can adversely affect biological tissues, causing local hemostasis, morphological changes, dehydration, and in some cases even tissue necrosis. The aim of this study was to study the effect of Omnipaque®300 and fructose solutions of various concentrations and exposure times on the intact skin using confocal Raman microspectroscopy. It was shown that the application of each of these OCAs on intact skin for 5 min also leads to an appreciable OC effect. The increase in OC was achieved using a mixture of Omnipaque®300 with DMSO; it was shown that the optical properties of the skin can be controlled at a depth of about 80 μm.

Keywords: confocal Raman microspectroscopy, optical clearing, skin, stratum corneum, Omnipaque®, fructose, penetration depth

1. INTRODUCTION

During the last years, the interest to the development and application of optical methods for clinical functional imaging of physiological processes, for diagnostics and therapy of cancer and other diseases is permanently growing [1,2]. It is caused by the unique information, relative simplicity, safety, and sufficiently low cost of optical instruments, as compared, to e.g., X-ray computer tomography (CT) or magnetic resonance imaging (MRI). However, the main limitation of optical diagnostic methods, including optical diffusion tomography, optical coherence tomography (OCT), confocal Raman microscopy (CRM), reflection spectroscopy, etc., is the strong scattering of light in biological tissues and blood,strongly reducing the contrast, spatial resolution, and probing depth [2-4].

A simple and efficient method of solving the problem of increasing the depth and quality of tissue structure imaging, as well as of increasing the precision of spectroscopic information from the deep tissue layers and blood, is the temporal and reversible reduction of the tissue-based light scattering by application of optical clearing agents (OCAs) [2, 4]. The main origin of the high scattering of skin is the presence of water in the intercellular/interstitial space, which refractive index is lower (1.33) in comparison to that of solid skin components, such as proteins (1.47) and lipids (1.47) [2]. The stratum corneum (SC) is a highly scattering structure due to the strong refractive index mismatch between dense dead corneocytes and intercellular substance with a low index of refraction due water content and air bubbles in the SC superficial cell layers [2,5-7].

Many agents have been found to be effective for local optical clearing (OC) *in vivo*, such as glycerol, dimethyl sulfoxide (DMSO), iohexol (Omnipaque®), propylene glycol, polyethylene glycol, glucose and fructose solutions [2,4,8-13].

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Despite the large number of publications, the problem of finding the most effective OCA, exposure time to the skin, as well as application of physical and chemical enhancers of its penetration, is still actual. Not enough attention has been paid to study the effect of OCAs on the parameters and components of the skin *in vivo*, on the safety of topical application of OCAs to the skin. It was shown that prolonged use of highly concentrated OCAs can have a negative effect on the skin (local hemostasis, dehydration, etc.). Some OCAs serve as penetration enhancers and can cause a change in the skin morphology due to the dissociation of collagen fibers [14-16] and the physiological effect of dehydration on the vascular network of the dermis. Thus, in order to avoid or reduce the negative effect on the skin, it is critical to find the optimal OCAs, their concentration, allowable exposure time and number of applications on the skin *in vivo*. Often, to increase the effectiveness of OC, the SC is completely or partially removed. It is a slightly invasive method. Thus, it is important and relevant to investigate the efficacy of intact skin OC, i.e. without affecting the unity of its structure.

For OC, the dermis is of great interest. Recent *ex vivo* studies using the CRM method have shown that OCAs of various types and concentrations can lead to dehydration of collagen [17], which is associated with a decrease (displacement by the action of OCAs) of weakly and strongly bound water [18], which describes the effect of OC - a decrease in the gradient of refractive indices due to the outflow of water in the dermis, leading to a decrease in skin scattering. These measurements were carried out *ex vivo* on porcine skin with a partially removed SC. In this regard, of interest are the results of data from *ex vivo* measurements without a preliminary effect on the SC of intact skin, which should show a real picture of the effects of OCA on the SC barrier, on dehydration below the underlying skin layers including the dermis and on the effectiveness of the OC method.

The aim of this work was to study the effect of topically applied Omnipaque® and fructose solutions at various concentrations and exposure times to the intact skin *ex vivo*.

2. MATERIALS AND METHODS

Pig ear skin (pig age ≈6months) was chosen as an appropriate *ex vivo* model of human skin [19]. Pig ears were received from a pig farm no later than 4 hours after slaughter. The procedure for preparing the skin for measurements included cleaning using cold running water, removing water residues with a paper towel and removing bristles with scissors that did not violate the structure of the SC. For measurements, pig ears were stored in a refrigerator at a temperature of +5 °C. The maximum storage time was 5 days. Before starting the measurements, the skin adapted to the environmental conditions of 21 °C in the laboratory for 30 minutes. Ten different pig ears were used in the experiment. Each study was carried out using 3 skin samples without visual damage. Skin samples were excised with a scalpel and separated from the cartilage of each individual experiment (sample size ≈1×1 cm^2, thickness ≈1 mm). Further, skin samples were placed on a glass slide and a 12 mm paper disc was placed on the skin surface onto which 100 µL of an OCA was applied from the skin contact side (see Table 1). The exposure time was 5, 15, 30, and 45 minutes. After removing the paper disc, excess agent was removed with filter paper. Control measurements were performed for the skin without the application of an OCA.

Table 1.Refractive index of used OCAs

OCA	Refractive index at 785 nm
DMSO 100%	1.4699
Omnipaque® 100%	1.4327
Omnipaque 90%+DMSO 10%	1.4364
Water-ethanol fructose 25%-25%-50% solution	1.3850

Ex vivo measurements on the skin of a pig ear were carried out on a model 3510 SCA confocal Raman microscope (River D International B.V., Rotterdam, Holland). To obtain Raman spectra in the 400–2200 cm^{-1}wavelength range, irradiation with a wavelength of 785 nm (Innovative Photonic Solutions semiconductor laser, Monmouth Junction, New Jersey, USA) was used. The optical power on the skin surface was 20 mW (laser power on the skin surface was

1.1 J/cm^2), the exposure time was 5 sec. The measurements were provided near the skin surface and the Raman signal profile was measured to a depth of 90 μm with a step of 5 μm. To accurately determine the position of the skin surface, the first 20 μm were measured with a step of 2 μm. The skin surface was determined to be at the position where the Raman line at 1665 cm^{-1} reached half of its maximal intensity [20]. Further, all depth values were adjusted relative to this position. For each skin sample, six depth profiles from different positions of the sample were measured. The depth of OCA penetration into the skin was calculated using the Skin Tools program, which is supplied with the CRM. The methodology for determining the depth of penetration of OCA into the skin is based on the determination of the relative content of the OCA at various depths of the SC by analyzing the Raman spectral contribution of the OCA using model Raman spectra of the components of the SC [21].

Pre-processing of Raman spectra included subtraction of fluorescence background near the Raman lines at 1003 and 1665 cm^{-1} using linear functions.

3. RESULTS AND DISCUSSION

The Raman spectra of the used OCA and untreated *ex vivo* pig skin on depth 20 μm are shown in Fig. 1.

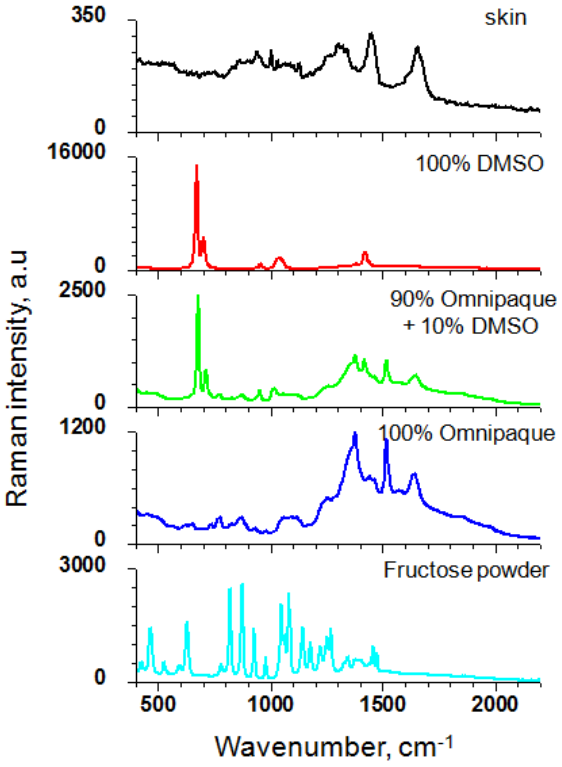

Figure 1.Raman spectra of porcine skin (depth 20 μm) and used OCAs

In Figure 1 in spite of the initial superposition of the skin and OCAs (DMSO, Omnipaque®, fructose) based Raman bands, areas of minimal Raman band superposition, for example, the skin bands at 1003 and 1665 cm^{-1}, can be seen. Thus, to determine the effectiveness of OC, the intensity of these Raman bands was analyzed.

The penetration depth of OCAs into the skin differs and is presented in Table 2. Taking into account that the average thickness of the SC of pig skin is equal to 18 ± 2 μm [22], we can conclude that DMSO penetrates through the SC at certain exposure times, which is consistent with literature data [23]. Among other OCAs used, a solution of water-

ethanol fructose 50% solution at all exposure times penetrated through the SC. When 10% DMSO is added to the Omnipaque® solution, the resulting solution almost saturates the entire SC, which is most likely due to the penetration enhancement effect of DMSO.

Table 2. The penetration depth of the used OCAs in the skin

OCA	**Exposition time to the skin, min**			
	5	15	30	45
	Penetration depth, μm			
DMSO 100%	14±4	27±8	30±5	33±8
Omnipaque® 100%	12±3	11±2	10±2	11±1
Omnipaque®90%+DMSO 10%	17±4	15±3	18±4	18±2
Water-ethanol fructose 25%-25%-50%solution	25±8	23±9	20±7	27±7

Figure 2 shows the calculated OC efficiencies for the applied OCAs used for two Raman bands at 1003 cm^{-1} (left) and 1665 cm^{-1} (right), corresponding to phenylalanine/urea and amide I vibrations, respectively.

Firstly, it was noted that when using pure DMSO, the highest OC efficiency was achieved. It is known that DMSO destroys the lipid barrier of the SC [23] and changes the interfibrillar distance between the collagen fibers of the dermis [24]. For this reason, DMSO is most often used as a chemical enhancer of the penetrating power of OCA and much less often as an independent OCA.

It was shown that the use of each OCA on intact skinfor5 min also lead to a noticeable OC effect. The increase in OC efficiency was achieved using a mixture of OCAs with DMSO. Moreover, when using a water-ethanol fructose 25%-25%-50% solution and a solution of 90% Omnipaque®+ 10% DMSO, the OC efficiency increases up to4.1 and 4.2at a depth of 80 μm, respectively.

In compare with data [17], it was found 2-fold increasing OC efficiencies of Omnipaque® 100% (exposure time 30 min) for 1003 cm^{-1} (0, 40 and 80 μm).

According to the previous studies of Omnipaque® clearing efficiency during skin treatment and current studies of OCAs influence on bound and unbound water loss in skin, it can be suggested that the efficiency of OC is directly related with the one of the main mechanisms of OC (dehydration) and depends on the amount of water loss during the OCA treatment. But, prolonged application of OCAs causing strong dehydration can result in negative effects on living tissues. In this way, tradeoff between efficiency of OCAs and their safety should be found for clinical *in vivo* measurements. The weakly and strongly hydrogen bound water types are preferentially involved in the OCA induced water flux in the skin, and thus, are responsible for OC efficiency.

Recently it was shown that the application of Omnipaque® 100% during 30 minutes does not result in significant changes in skin full water profile and only a tendency to reduction (skin dehydration)has been observed (4.4%) [18]. Among the water types in the skin, the following reduction was observed in concentration of weakly bound (4.6%), strongly bound (3.4%), tightly bound (12.9%) and unbound (5.9%) water types on average for the 40 to200 μm depths, post application of Omnipaque® (30 minutes). As most concentrated in the skin, weakly and strongly bound water types are preferentially involved in the OCA-induced water flux in the skin, and thus, are responsible for OC efficiency.

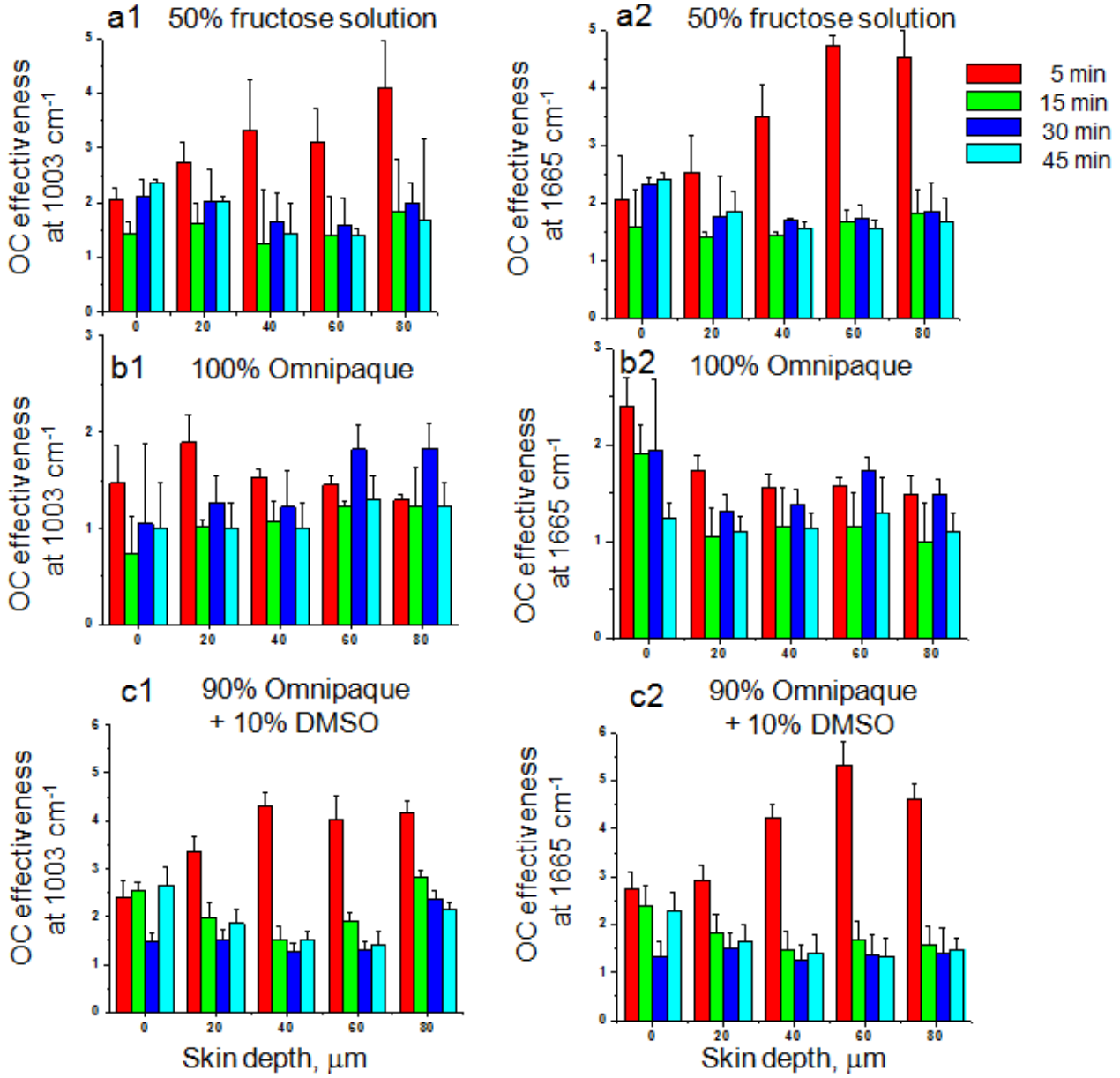

Figure 2 The effectiveness of OC for skin samples treated with water-ethanol fructose 25%-25%-50% solution (a); 100% Omnipaque® (b); a solution of 90% Omnipaque® + 10% DMSO (c) for different exposure times (5, 15, 30, and 45 min) at 1003 cm^{-1} (a1, b1, c1) and at 1665 cm^{-1} (a2, b2, c2).

4. CONCLUSIONS

Usually, to provide a more effective OC in skin the SC is partly removed. However, this procedure is slightly invasive. Thus, in this *ex vivo* study intact skin without removing/disturbing the SC was conducted. The effect of local application of Omnipaque® and fructose solutions of different concentrations on the effectiveness of OC was investigated. It was shown that the use of OCAs on intact skin also leads to a reasonable OC effect. The effectiveness of OC is most likely associated with the displacement of water from the deep regions of the SC.

The maximal efficiency, increase of around 4-foldat a depth of 80 µm, is achieved after5 minutes exposure of water-ethanol-fructose 25%-25%-50% solution and solution of 90% Omnipaque® + 10% DMSO.

It has been demonstrated that despite the small thickness of the SC, it plays a decisive role in OC of the skin and an increase in the depth of its probing up to the dermis layer, which is caused by an almost ten-fold bigger initial scattering coefficient of the SC compared to the epidermis and dermis.

The increase of OC efficiency was achieved at a depth of 80 μm (approximately 2–4 fold) using a mixture of OCAs and DMSO/ethanol for all exposure times (Fig. 2a and 2c). The maximal efficacy of OC of 100% Omnipaque® (Fig. 2b) was 1.8 at 30 min exposure time (depth 80 μm).

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