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Authors

Bui, Albert K
McClure, R Anthony
Chang, Jennell
et al.

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Revisiting optical clearing with dimethyl sulfoxide (DMSO)

Albert K. Bui¹, R. Anthony McClure^{1,2}, Jennell Chang¹, Charles Stoianovici³, Jason Hirshburg⁴, Alvin T. Yeh⁴, and Bernard Choi^{5,6}

¹School of Biological Sciences, University of California, Irvine, California 92697

²Department of Neurobiology, University of California, Irvine, California 92697

³Department of Chemistry, University of California, Irvine, California 92697

⁴ Department of Biomedical Engineering, Texas A&M University, College Station, Texas 77843-3120

⁵Department of Biomedical Engineering, University of California, Irvine, California 92697

⁶Beckman Laser Institute, University of California, Irvine, California 92697

Abstract

Functional optical characterization of disease progression and response to therapy suffers from loss of spatial resolution and imaging depth due to scattering. Here we report on the ability of dimethyl sulfoxide (DMSO) alone to reduce the optical scattering of skin. We observed a three-fold reduction in the scattering of skin with topical DMSO application. With an *in vivo* window chamber model, we observed a three-fold increase in light transmittance through the preparation and enhanced visualization of subsurface microvasculature. Collectively, our data demonstrate the potential of DMSO alone to mitigate effects of scattering, which we expect will improve molecular imaging studies.

Keywords

glycerol; hyperosmotic agents; collagen dissociation; collagen solubility; integrating spheres

INTRODUCTION

Recent data by Yeh et al. (1) and Hirshburg et al. (2,3) suggest that a chemical agent's optical clearing potential is strongly tied to its ability to screen non-covalent, hydrophilic, intermolecular attractive forces resulting in destabilization of high-order collagen structures. Unfortunately, these agents typically possess hydrophilic properties, and hence are unsuitable as topically-applied agents due to their inability to penetrate through the lipid-rich stratum corneum. The primary objective of our study was to evaluate the optical clearing potential of DMSO (Figure 1) when applied topically to *in vitro* and *in vivo* skin. To achieve this objective, we employed multiple optical spectroscopy methods to evaluate rapidly various chemical agent formulations.

MATERIALS AND METHODS

Chemical Agents

Dimethyl sulfoxide (DMSO, EMD, 99.9% purity, 14M) solutions were prepared with either isotonic saline (Phoenix Pharmaceuticals) or ethanol (Gold Shield, 200 proof) as diluents. Glycerol (Sigma-Aldrich) solutions were prepared with ethanol as the diluent. To study the potential of chemical penetration enhancement, we evaluated glycerol/limonene solutions.

Glycerol (7M and 14M) was combined with 5% (w/v, with ethanol as the diluent) limonene (98% purity). Isotonic saline was used as a negative control since it had no expected optical clearing potential (OCP) (4).

Skin Preparation

Cryopreserved, dermatomed human skin (Science Care, Phoenix, AZ) was thawed to room temperature (~26°C). With a single-edged razor blade, the skin was cut into ~2.5 cm × 2.5 cm samples. The thickness of each sample was measured by placing it between two glass slides, clamping the preparation with binder clips to provide consistent compression and uniform thickness, and measuring the preparation thickness with a micrometer (Mitutoyo, City of Industry, CA). Sample thickness ranged between 1.2 and 1.5 mm. To minimize systematic error and maximize repeatability of the measurement method, we recorded the preparation thickness after one click of the fine adjustment screw on the micrometer. By calculating the difference between the thickness of the preparation and the slides, we determined thickness of each sample.

Reflectance Spectroscopy

Reflectance spectroscopy was used to assess qualitatively whether or not individual chemical agents possessed OCP. We selected this method for initial evaluation because it permitted rapid screening of multiple agents. A reflectance probe (Ocean Optics, Dunedin, FL) equipped with multiple source and detector fibers was used to deliver light from a tungsten halogen source to and collected remitted light from each skin sample. The samples were placed on a black plastic plate with the epidermal side facing up. O-rings and Franz cell caps were placed on top of each sample to serve as a chemical well and ensure that each sample was saturated with agent. An online random number generator (<http://www.randomizer.org>) was used to assign the agents to each skin sample as well as to randomize experimental order. One mL of agent was added to each well at each time point (0, 60, 120, 180, and 240 minutes).

At each time point, each O-ring and Franz cell cap was removed and any remaining agent removed from the skin using a Kimwipe tissue. The probe was placed on the epidermal side of the skin with slight pressure to ensure minimal background noise and the optical power of the collected reflected light recorded. For all measurements, the integration time was 150 ms. Since each skin sample was placed on top of a black absorbing plate, agents with OCP were identified by a decrease in reflectance at the 600 nm wavelength. After each measurement, the O-ring and Franz cell cap were put back on the skin sample and the appropriate agent was reapplied to each sample. This procedure was repeated at each time point as described above.

Statistics and Data

Data were analyzed with InStat (Graphpad, San Diego) using a repeated measures analysis of variance test. Tukey's test was used as a multiple comparison post test if significance were obtained. P-values less than 0.05 were deemed as significant changes.

Quantitative Evaluation of Agent OCP

Based on the results of the rapid-screening, epidermal-application experiments described above, the OCP of promising agents was quantified. New skin samples were prepared and thicknesses measured using the methods described above. Total light transmittance and diffuse reflectance were measured using an integrating sphere method (5). After each sample was clamped between two glass slides, the sample/slide combination was irradiated by a collimated 785 nm laser diode (BW TEK, Canada). Transmittance and reflectance values

were measured by placing the sample/slide combination at either the entrance or exit port, respectively, of the integrating sphere (Labsphere, North Sutton, NH). System calibration and measurement procedures followed steps established by Choi et al. (4).

After baseline (0 min time point) transmittance and reflectance measurements, each sample was removed from the glass slides and placed upon Franz diffusion cells with the epidermal side facing up. The receptor compartment of each cell was filled with isotonic saline and encased in a flow jacket with circulating water heated to 37°C to mimic *in vivo* conditions. Any air bubbles were removed to ensure complete contact between saline and the dermal side of the sample. Once the donor compartment reservoir was clamped on top of the sample, one mL of agent was applied to the reservoir and Parafilm used to cover the donor compartment cap to maintain occlusive OCA application conditions. Chemical assignment and experimental order were determined with a random number generator as described above.

At each ensuing measurement time point (60, 120, 180, and 240 min), the samples were removed from the diffusion cells and excess agent was removed with a Kimwipe tissue. Sample thickness and transmittance and reflectance measurements were obtained as described above. After these measurements, each skin sample was remounted within its cell and a fresh volume of the appropriate chemical reapplied.

OCP was assessed by incorporating skin thickness, total transmittance, and diffuse reflectance into an inverse adding-doubling algorithm to estimate skin reduced scattering coefficients. A decrease in the coefficient indicated that the formulation had OCP. The reduced scattering ratio (RSR) of each agent was quantified as the ratio of reduced scattering coefficients prior to and at specific time points after agent application (2,3). All data were analyzed as described above.

Mouse Dorsal Window Chamber Model

To study *in vivo* optical scattering dynamics, C3H mice with surgically installed dorsal window chambers were used. All procedures were performed as approved by the University of California, Irvine, Animal Care and Use Committee. The surgical protocol is similar to one used previously (6). Briefly, titanium window chambers were sutured to the stretched dorsal skin of each rodent. One full thickness of skin was removed from a 12-mm-diameter region, exposing a subdermal microvascular network of the opposing full thickness of skin. A glass cover slip was placed over the exposed subdermal tissue to impede dehydration.

In Vivo Optical Measurements

A 6.9-mm diameter rubber gasket was placed over the epidermal side of the window chamber skin. A Hilltop chamber with a 0.7 cm diameter hole removed from the center was placed over the window and gasket to minimize agent leakage during the experiments. After anesthesia, the animal was placed sideways on a custom stage with the epidermal side of the window chamber facing upwards. DMSO (14M) was applied topically and removed at specific timepoints (0, 5, 10, and 20 min after agent application) for data collection. Laser light ($\lambda = 785$ nm) transmittance was measured with a spectrometer equipped with an integrating sphere. After 20 min DMSO application, isotonic saline was applied to the epidermal side in an attempt to reduce tissue transparency. Color diffuse reflectance images also were collected at each timepoint. The window chamber was transilluminated with a light source contained within the integrating sphere and color images acquired. A transparency with a grid of black lines was placed directly beneath the window chamber, to serve as a known, inert target for qualitative interpretation of optical clearing.

Collagen Solubility

Collagen solubility measurements were conducted similar to previous studies (2,3,7,8). Self assembly of soluble rodent tail type I (pH ~ 3) collagen (BD Biosciences, San Jose, CA) was carried out in 1N phosphate buffered saline (PBS). Soluble collagen was mixed with chemical agents (glycerol, DMSO + PBS, DMSO + EtOH) resulting in solutions of 3.13 mg/ml collagen in 0.4M – 1.6 M chemical agent. The pH was adjusted to physiological level (7.4) inducing collagen fibrillogenesis and incubated for 48 hours. After completion of fibrillogenesis, the solutions were centrifuged (12,000 rpm for 15 min) to remove formed collagen fibril precipitate leaving soluble collagen in the supernatant. Collagen solubility was measured using optical absorbance (USB2000, Ocean Optics, Dunedin, FL).

RESULTS

Reflectance spectroscopy was used as a rapid screening method to assess qualitatively whether or not individual chemical agents possessed optical clearing potential (OCP). Topical application of 14M DMSO and 10.5M DMSO/EtOH *in vitro* showed OCP while glycerol/limonene showed no OCP (Figure 2A). 14M DMSO and 10.5M DMSO in ethanol showed significant OCP ($p = 0.001$ and 0.0004 , respectively) when applied topically to human skin *in vitro*. Interestingly, the mixture of 10.5M DMSO in saline produced an initial exothermic reaction, but showed no significant OCP ($p = 0.014$). With subdermal application of 14M DMSO and 10.5M solutions of DMSO in either ethanol or saline, we observed OCP (Figure 2C), suggesting that the reaction between DMSO and saline negates the ability of DMSO to penetrate the stratum corneum but does not affect its ability to alter skin optical scattering properties. We also performed topical application experiments with glycerol formulations. Limonene was used in specific experiments due to its known chemical penetration enhancement capabilities (9). However, our data demonstrate that the glycerol/limonene chemical conditions did not show any significant OCP (Figure 2B).

Based on the rapid screening data, 14M DMSO and 10.5M DMSO in EtOH were chosen for quantitative evaluation. Figure 2D summarizes the results for the quantitative evaluation. 14M and 10.5M DMSO in ethanol had significant OCP ($p = 0.002$ and 0.001 , respectively) while 10.5M DMSO in saline and saline alone had no significant OCP ($p = 0.134$ and 0.332 , respectively). A mixture of DMSO and saline once again produced an exothermic reaction.

Topical application of DMSO to *in vivo* rodent skin demonstrates feasibility of *in vivo* optical clearing. From light transmittance measurements, we observed an increase in light transmittance through full-thickness skin in a dorsal window chamber, suggesting a reduction in the degree of skin turbidity (Figure 3A). Subsequent topical saline application led to a progressive decrease in light transmittance, suggesting a reversal of the optical clearing effect. From color reflectance images taken in concert with the light transmittance measurements, we observe an increase in visibility of subsurface skin microvasculature (Figure 3B). Collectively, these results demonstrate the potential of a simple DMSO-based optical clearing method for enhanced microvascular imaging. It is possible that longer application periods may yield further increases in light transmittance and the degree of clarity of the microvasculature; such optimization experiments will be performed in future work.

The mechanisms underlying optical clearing remain an open discussion point. The initial proposed mechanisms included refractive index matching and skin dehydration due to the hyperosmotic nature of evaluated OCAs (10). However, data from one of our previous studies provided evidence that the OCP of various OCAs had no apparent correlation with either OCA refractive index or osmolality, suggesting that another mechanism exists (4). Our recent data provide compelling evidence that sugar alcohols such as glycerol can

destabilize collagen structure, and the degree of destabilization observed *in vitro* corresponds well with measured agent efficacy (1–3). Hence, we employed a collagen solubility experimental protocol (2,3) to determine if collagen destabilization effects (1) play a role in the underlying mechanism of the DMSO optical clearing effect. The present collagen solubility data (Figure 4) demonstrate that the presence of DMSO exhibits a weak concentration-dependent effect on the self assembly of collagen molecules into fibrils, similar to that observed previously with ethylene glycol (2). Furthermore, based on interpolation of our optical scattering data collected during 13.6M ethylene glycol application to human skin samples, we estimate a 3.5-fold scattering reduction, which is similar to the threefold reduction observed in this study (Figure 2D). Preliminary second harmonic generation imaging (Max Zimmerley, Bernard Choi, Eric Potma, unpublished data) of skin collagen structure with DMSO application demonstrate changes in collagen structure with DMSO application, to a lesser degree than that observed previously with glycerol (1). Collectively, the data in Fig. 4 and our previously-published collagen solubility data (2) suggest that the optical clearing potential of both sugar alcohols and DMSO is closely linked with the ability of these OCAs to destabilize collagen structure. Future experiments are planned to study this mechanism in greater detail.

DISCUSSION

Optical clearing has been used with *in vitro* preparations to demonstrate enhanced optical spectroscopy (11–13), fluorescence imaging (14), and bioluminescence and chemiluminescence imaging (15,16). Use of known optical clearing agents such as glycerol, propylene glycol, and glucose, can improve the contrast and imaging depth for confocal and multiphoton microscopy (17–19) and optical coherence tomography (10,20,21). The potential benefits of optical clearing to laser therapy of subsurface targets such as tattoo pigments and microvasculature have been demonstrated (22–24). Unfortunately, these studies have been limited primarily to agent application directly to the dermis, thus bypassing the stratum corneum barrier. In general, agents with demonstrated OCP are unable to penetrate in a timely fashion the stratum corneum skin layer. Thus, optical clearing agent penetration via topical application to skin has been the subject of intense study, with some promising results. Methods used to enhance OCA diffusion into skin have included focal laser-induced thermal damage of the stratum corneum (25–27), microneedle rollers (28), and dermabrasion (29). Demonstration of the clinical potential of a topically applied amphiphilic agent for optical clearing has been performed (21,22), although efficacy has been found to be highly variable [unpublished data]. Chemical penetration enhancers are a commonly studied method to improve the penetrance of drugs (9,30). Based on optical transmittance data acquired with *in vitro* porcine skin, Jiang and Wang (31) and Moulton et al. (32) concluded that agents such as DMSO and oleic acid are viable penetration enhancers for known OCAs such as glycerol and polyethylene glycol.

Due to the known penetrance of DMSO into intact skin and known OCP with dermal application (4,11), we set out to study its ability to serve as a simple, effective optical clearing agent. Collectively, our *in vitro* and *in vivo* results provide compelling evidence that DMSO *by itself* is an effective topical optical clearing agent, reducing the degree of skin optical scattering by a factor of three and improving our ability to visualize subsurface blood vessels.

However, DMSO currently is a shunned chemical agent due to its purported systemic toxicity. To assess the viability of DMSO as an optical clearing agent, we reviewed the DMSO safety literature. We determined that the majority of toxicological studies involving DMSO were conducted prior to 1980 and did not provide definitive proof of the local and systemic effects of the chemical. In addition, the scientific community and the FDA

have expressed mixed evaluations of DMSO. The most comprehensive independent study completed on DMSO was performed in 1972 by the National Academy of Sciences. Based on data from this study, the FDA ultimately concluded that clinical studies of DMSO are still warranted to demonstrate both the efficacy and safety of DMSO (33). We believe that the FDA's stance is insufficient to reject outright the potential of DMSO as a topically applied agent, as data from toxicity studies are oftentimes conflicting and hence not fully understood. The expected benefits of optical clearing to studies employing molecular sensing of subsurface events warrant future research on DMSO systemic toxicity.

Ideally, the application time of any OCA needed to reduce tissue optical scattering should be as short as possible. Our data (Figure 2D) suggest that a twofold increase in optical penetration depth could be achieved with a 120-min application of 14M DMSO. Such application times are easily achieved with occlusive, adhesive wells such as Hill Top Chambers (Hill Top Research, Miami, OH).

Rapid developments in optical imaging and microscopy have led to a growing number of studies employing this technology. These studies focus primarily on use of molecular absorption and fluorescence characteristics as a source of contrast, and optical scattering has been viewed as an unavoidable restriction limiting the ultimate potential of optical-based methods. The optical clearing approach to improve characterization of subsurface molecular and cellular processes uniquely addresses the restrictions imposed by optical scattering on accurate localization and quantification of the optical signals of interest (i.e., fluorescence, bioluminescence, etc.). Application of optical clearing is expected to enable clinicians and scientists employing optical methods to interrogate and target previously unattainable biological tissue features *in situ*, extending the range of suitable applications for biophotonics.

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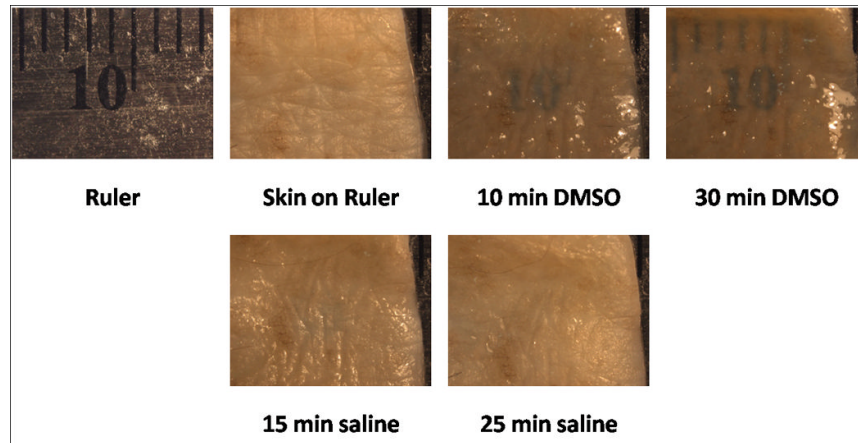


Figure 1. Representative example of optical clearing for enhanced optical imaging
Dermatomed, cryopreserved human skin samples were immersed in 14M DMSO for 30 min. (TOP ROW) Visualization of an opaque metal ruler that was placed underneath the samples immersed in DMSO for different periods of time demonstrates the dynamic and dramatic changes in skin transparency. Within 10 min of immersion in DMSO, features of the ruler underneath the skin were easily discernible. (BOTTOM ROW) To demonstrate the reversible nature of optical clearing, the sample was then placed in phosphate buffered saline for 25 min, after which scattering of the sample returned.

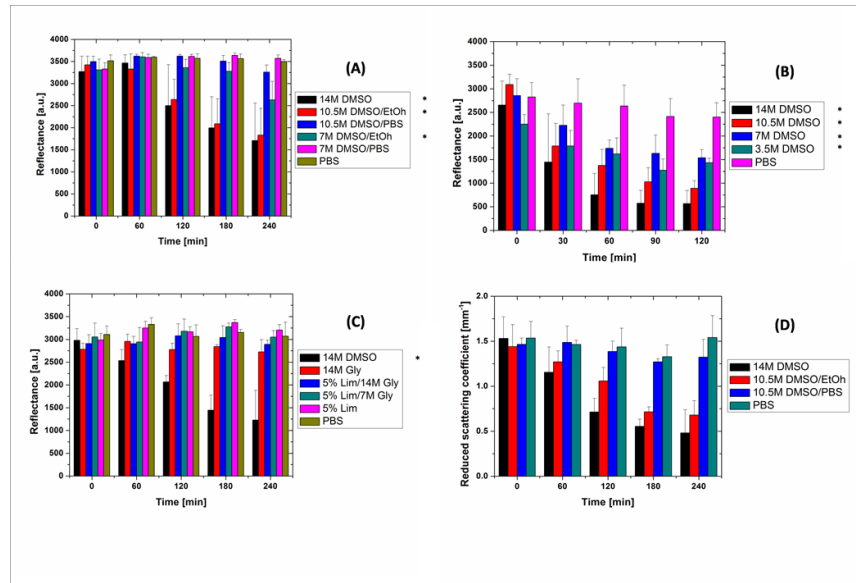


Figure 2. Summarized data from rapid-screening reflectance spectroscopy measurements

(A) Time-resolved reflectance during topical application of 14M DMSO, 10.5M DMSO in ethanol (EtOh), 10.5M DMSO in phosphate-buffered saline (PBS), 7M DMSO/EtOh, 7M DMSO/PBS, and PBS (negative control). 14M DMSO and 10.5M DMSO in EtOh exhibited significant OCP ($p = 0.001$ and 0.0004 , respectively) while 10.5M DMSO in PBS and PBS alone did not exhibit significant OCP ($p = 0.14$ and 0.63 , respectively). Weaker DMSO solutions (3.5M and 1.4M, in either EtOh or PBS) did not exhibit significant OCP (data not shown). (B) Time-resolved reflectance during *subdermal* application of 14M DMSO, 10.5M DMSO/PBS, 7M DMSO/PBS, 3.5M DMSO/PBS, and PBS. All DMSO/PBS agents exhibited OCP ($p = 0.0002$, 0.0001 , 0.001 , and 0.0055 for 10.5, 7, and 3.5M solutions, respectively) with subdermal application, as opposed to no significant OCP with topical application [data in (A)]. Saline did not exhibit OCP ($p = 0.1991$). (C) Time-resolved reflectance during topical application of 14M DMSO, 14M glycerol (Gly), 14M Gly in 5% limonene (Lim), 5% Lim/7M Gly, 5% Lim, and PBS. No significant OCP was observed for any of these evaluated agents ($p = 0.400$, 0.283 , 0.210 , 0.430 , and 0.429 , respectively). Note that the data shown for 14M DMSO applications in (A) and (C) differ because they represent experiments performed at different sessions. (D) Summary of reduced scattering ratio (RSR) extracted from data collected during topical application of 14M DMSO, 10.5M DMSO/EtOh, 10.5M DMSO/PBS, and PBS. 14M DMSO and 10.5M DMSO in EtOH exhibited significant OCP ($p = 0.002$ and 0.001 , respectively) while 10.5M DMSO in saline and saline did not exhibit any significant OCP ($p = 0.134$, and 0.332 , respectively). For all graphs in this Figure, the symbol * represents an experimental condition in which a significant OCP ($p < 0.05$) was observed.

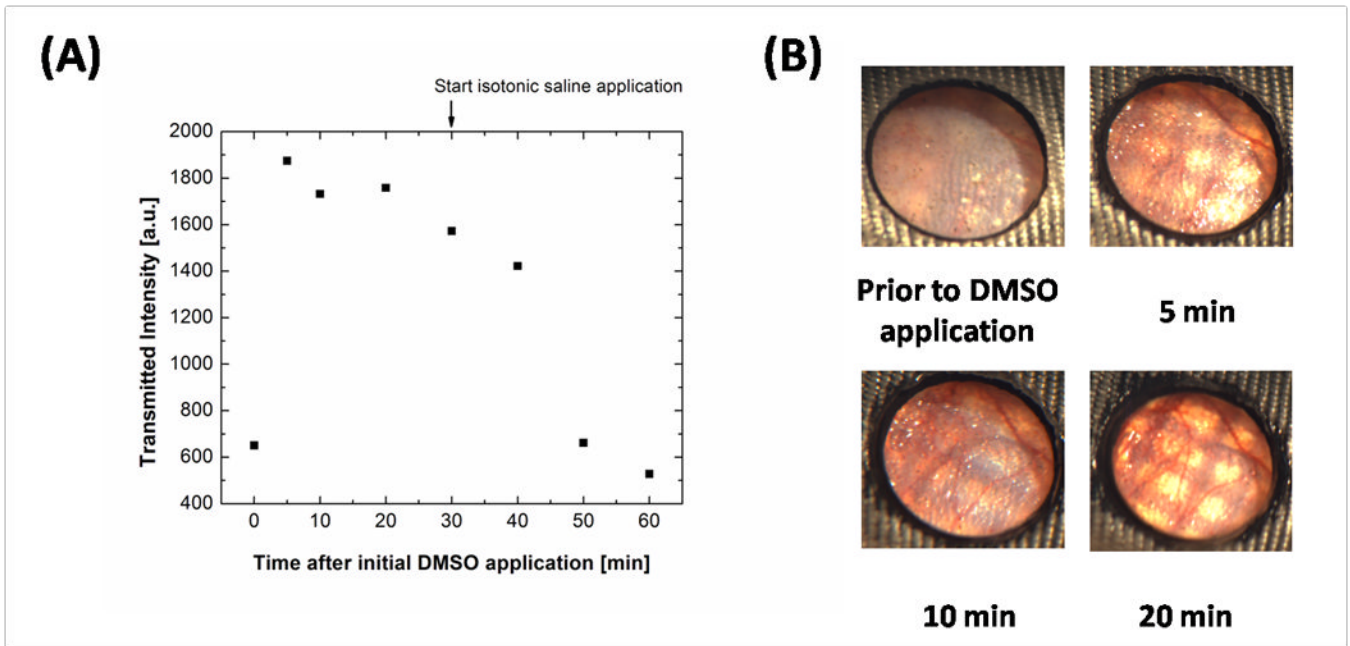


Figure 3. Representative (A) light transmittance and (B) color reflectance images during topical application of 14M DMSO demonstrate a reduction in skin turbidity and enhanced visualization of subdermal blood vessels

A dorsal window chamber was surgically installed on an adult C3H mouse and DMSO applied to the epidermal side of the skinfold. The increase in light transmittance (seen in (A)) during the initial 20 min coincided with enhanced visualization (B) both of subdermal microvasculature and a custom resolution target placed directly below the glass coverslip overlying the subdermal side of the skinfold. With subsequent topical delivery of isotonic PBS, the transmittance decreased to baseline (i.e., before topical DMSO application).

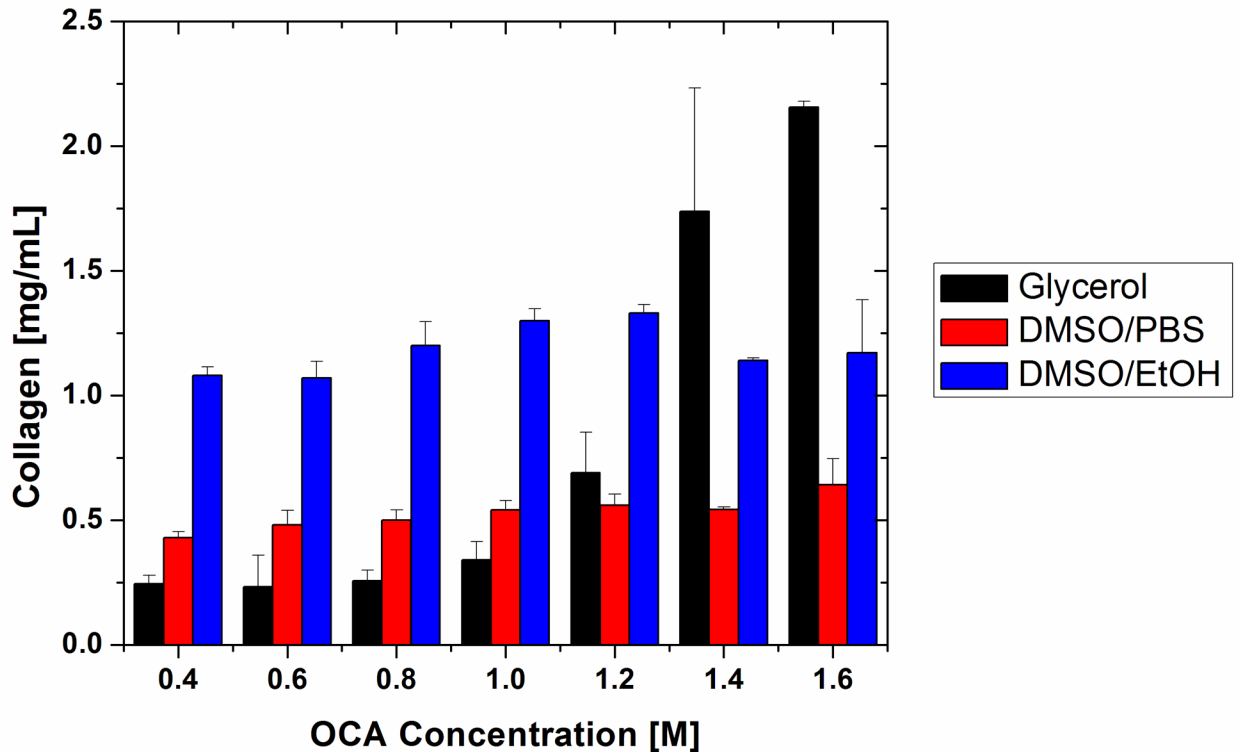


Figure 4. Over the evaluated agent concentration range, DMSO exhibits a concentration-dependent inhibition of collagen self assembly into fibrils

A t-test of the DMSO-PBS data results in a slope significantly different from zero ($p < 0.0001$). Solutions of collagen molecules (3.13 mg/mL) were incubated at physiological pH and temperature; this protocol is known to result in spontaneous self assembly of these molecules into collagen fibrils. Addition of glycerol to these solutions is known to inhibit the self assembly process; hence, addition of progressively higher glycerol concentrations results in higher concentrations of collagen molecules remaining in solution (black bars in Figure).