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Journal

Lasers in Surgery and Medicine, 39(2)

ISSN

0196-8092

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Publication Date

2007-02-01

DOI

10.1002/lsm.20417

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Correlation Between Collagen Solubility and Skin Optical Clearing Using Sugars

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Background and Objective: Light scattering from collagen within skin limits light-based therapeutics while increasing the risk of epidermal thermal injury. Specific chemicals show the ability to reduce light scattering by reversibly altering the optical properties of skin. This study examines the correlation between collagen solubility and the optical clearing potential (OCP) of sugars and sugar-alcohols using *in vitro* rodent skin.

Materials and Methods: Collagen solubility in dextrose, fructose, sucrose, and sorbitol was measured using near-UV spectroscopy. Light transmittance, reflectance, and rodent skin thickness were measured (giving skin reduced scattering coefficient) before and after exposure of the dermal surface to sugars and sugar-alcohols. OCP was calculated as the ratio of reduced scattering coefficients before and after exposures.

Results: Dextrose, fructose, sucrose, and sorbitol had at least twice the collagen solubility and twice the OCP as compared to glycerol. In general, collagen solubility correlated with each agent's ability to optically clear rodent skin.

Conclusion: These results demonstrate that sugar and sugar-alcohol interaction with collagen are a primary event in tissue optical clearing. *Lasers Surg. Med.* 39:140–144, 2007. © 2007 Wiley-Liss, Inc.

Key words: hyperosmotic agents; optical scattering; dermatology

INTRODUCTION

Efficacy of light-based therapeutics is limited by the turbidity of most biological tissues. For dermatologic applications, optical scattering predominantly from high-order collagen structures within the dermis [1,2] limits the effective light dose and depth at which treatments can be targeted. These imposed limitations by the optical properties of skin can only be partially compensated by current cooling technologies aimed at attenuating epidermal injury induced by pulsed laser exposure.

Traditionally, the optical properties of skin [1,3] have been considered fixed. However, after tissue immersion in selected non-reactive chemical agents, a temporary reduction in light scattering has been demonstrated [4,5]. Previous studies have shown that a reduction in tissue

optical scattering can improve the efficacy of light-based therapeutics [6]. Optical clearing can be dramatic as tissue becomes visibly transparent and loses mechanical compliance. This process is reversible when upon subsequent immersion in isotonic saline, tissue turbidity, and mechanical properties return to their native states [7]. These “clearing” agents are benign, biocompatible and already in use as sweetening additives in foods and emollients in skin care products. Optical clearing agents have been investigated primarily in collagenous tissues and have been shown to be most effective when applied directly to the dermis of skin [5,6,8].

Selection of potential agents has been empirical rather than based upon fundamental understanding of chemical agent induced tissue optical clearing. Common properties of optical clearing agents such as sugars and sugar-alcohols have suggested refractive index matching with native tissue components, in particular collagen, and dehydration are possible mechanisms of tissue optical clearing [5,6]. Under this proposed mechanism, refractive index matching of the clearing agent to that of collagen would reduce light scattering leading to an increase in tissue transparency. Dehydration was also believed to play a role as reported chemical agents are hyperosmotic with respect to tissue. However, use of these properties as selection criteria does not predict agent optical clearing potential (OCP) [9,10] which suggests an incomplete understanding of chemical agent induced tissue transparency.

Previous studies have suggested that non-reactive molecular interactions of chemical agents with collagen

Contract grant sponsor: National Science Foundation Faculty Early Career Development (CAREER) Award; Contract grant sponsor: Arnold and Mabel Beckman Foundation; Contract grant sponsor: National Center for Research Resources at the National Institutes of Health; Contract grant number: RR01192; Contract grant sponsor: National Institutes of Health; Contract grant numbers: EB004846, AR47551, EB02495, AR48458; Contract grant sponsor: ASLMS USAF Travel.

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Accepted 4 September 2006

Published online 26 February 2007 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/lsm.20417

have a prominent role in tissue optical clearing [for a review see Reference [8]]. It has been shown at microscopic and ultrastructural length scales that glycerol, a prototypical optical clearing agent, destabilizes high-order collagen structures and that this effect is necessary for agent induced tissue transparency [11,12]. In addition, glycerol's non-reactivity with collagen facilitates reversibility of its optical clearing effects. Collagen destabilization suggests that the primary light scatterer in skin is reduced in size for optical clearing by screening non-covalent, hydrophilic, attractive forces (hydrogen bonding). These same forces drive collagen fibrillogenesis from solution and have been characterized in the presence of sugars and sugar-alcohols [13]. Following this reasoning, OCP of potential chemical agents should follow their ability to disrupt collagen fibrillogenesis. We introduced collagen solubility as a measure of a chemical agent's ability to screen non-covalent forces and correlated it with tissue optical clearing for a series of polyols [9]. Herein, we studied the relationship between collagen solubility and tissue OCP for mono- and disaccharides. We hypothesized that collagen solubility and OCP would have a significant positive correlation.

MATERIALS AND METHODS

Collagen Fibril Formation and Solubility

In vitro self-assembly of solubilized (pH \sim 3) rodent tail collagen I (BD Biosciences, San Jose, CA) into fibrils was carried out in phosphate buffered saline (PBS) (0.138 M NaCl, 0.0027 M KCl). Stock collagen solutions (4.26 and 4.27 mg/ml) were diluted to concentrations of 0.25, 0.75, 1.50, and 2.13 mg/ml. These solutions were mixed with chemical agents under study at various concentrations. Glycerol, sorbitol, dextrose, fructose, and sucrose (Sigma Aldrich, St. Louis, MO) were all reagent grade quality and used without further purification. Chemical structure representations of these chemical agents are shown in Figure 1. With addition of NaOH, the solutions were adjusted to physiological pH (\sim 7.4), inducing collagen

fibrillogenesis, and incubated at 37°C for 24 hours. After fibrillogenesis was complete, the solutions were centrifuged at 12,000 rpm for 15 minutes to separate fibrils (white precipitate) from the remaining collagen molecules (supernatant). Collagen solubility was measured by optical absorbance of the supernatant. A calibration curve was determined by measuring optical absorbance at 215–230 nm [14] (USB2000, Ocean Optics, Dunedin, FL) of a series of known collagen solutions at different concentrations. Our previous study used optical absorbance at 276 nm to measure collagen concentration in solution [9]. Measured collagen concentrations using either absorbance wavelength were consistent with each other.

Rodent Skin Preparation

Ex vivo rodent skin (3–6 weeks old) was cut into 1.5×1.5 cm² samples using surgical scissors. Subcutaneous fat was removed using a razorblade and rodent skin stored in $1 \times$ PBS at 4°C until experiments were performed less than 24 hours later. Skin thickness was measured using a micrometer (Mitutoyo, Aurora, IL) after the sample had been placed between two glass slides of known thickness. The slides were secured using binder clips to ensure constant pressure on each skin sample.

Tissue Optical Clearing Measurement

An integrating sphere (Labsphere, North Sutton, NH) was used to determine the transmittance and reflectance of 635 nm laser diode light in rodent skin samples [15,16]. The skin/glass combination was placed at the entrance and exit ports of the integrating sphere for transmittance and reflectance measurements, respectively. For transmittance measurements, the exit port was covered using a spectralon-coated port plug (99% reflectance). Light measurements were conducted using a silicon photodiode (Labsphere) connected to an oscilloscope (Tektronix, Beaverton, OR). System calibration was performed using neutral density filters and reflectance standards (Labsphere). The inverse-adding-doubling method was used to calculate the reduced scattering coefficient, μ'_s , for each skin sample before and after clearing agent application [16]. Volume matched solutions of each clearing agent were applied to the dermal side of the skin sample. The chemical agents were applied for a total of 45 minutes and removed carefully with Kimwipes™ prior to transmittance and reflectance measurements. Reduced scattering ratio (RSR), the ratio of μ'_s before and after clearing agent application, was used to quantify the reduction in tissue μ'_s and chemical agent OCP. Each data point is an average of at least four measurements.

Statistical Analysis

MATLAB software (The Mathworks, Natick, MA) was used to perform a one-way analysis of covariance (ANCOVA) test on the RSR data of rodent skin to test for statistically significant differences in the linear regression for each chemical agent. The overall RSR data were found to be dependent on chemical type (categorical variable) and concentration (continuous variable). The effect of each

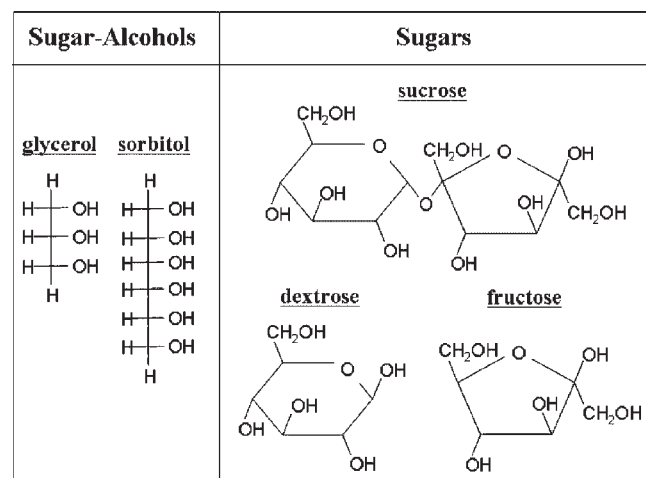


Fig. 1. Chemical structure representations of sugar-alcohols and sugars.

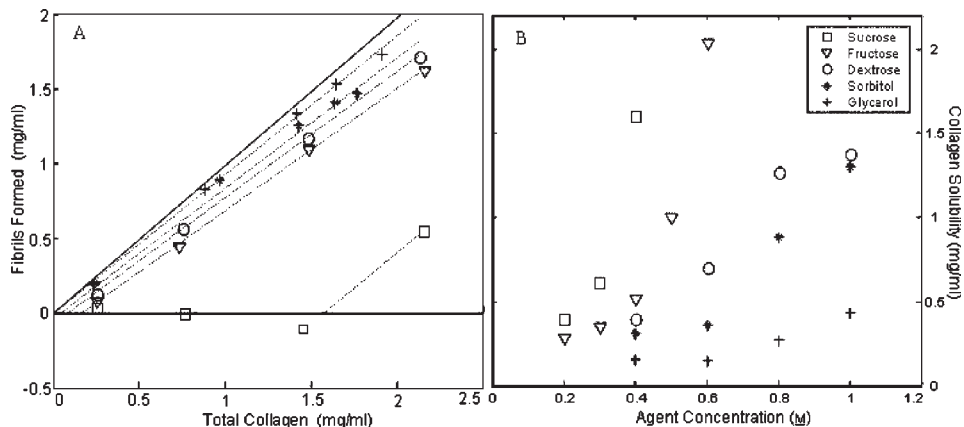


Fig. 2. Collagen fibrils formed (A) and solubility (B) for sugars and sugar-alcohols. A: Chemical agent concentration is 0.4 M. B: Initial collagen concentration is 2.13 mg/ml.

clearing agent on skin was assumed to be normally distributed. Analysis by chemical agent with the need to control for the covariate (concentration) called for the use of one-way ANCOVA. An F -test determined that RSR mean values were significantly different and t -tests were performed to compare the effects of the individual chemicals. The Scheffes multiple-comparisons procedure was used to correct for the error associated with the large number of comparisons [17].

RESULTS

Collagen fibrillogenesis and solubility are shown in Figure 2 for a series of sugars and sugar-alcohols. Fibril

formation from 0.4 M solutions of chemical agents is shown in Figure 2A and quantified by the difference in collagen content of supernatant before and after solution neutralization. For collagen in PBS only, fibrillogenesis efficiency is near unity (data not shown) and depicted by the bold line in Figure 2A. Sugars showed higher fibrillogenesis inhibition than sugar-alcohols. Sucrose was the largest molecule tested and had the greatest inhibiting effect on fibrillogenesis. Fructose, dextrose, sorbitol, and glycerol showed decreasing inhibition of collagen fibrillogenesis.

Collagen solubility of chemical agents is shown in Figure 2B and quantifies the amount of collagen remaining in solution after fibrillogenesis. Our previous study found

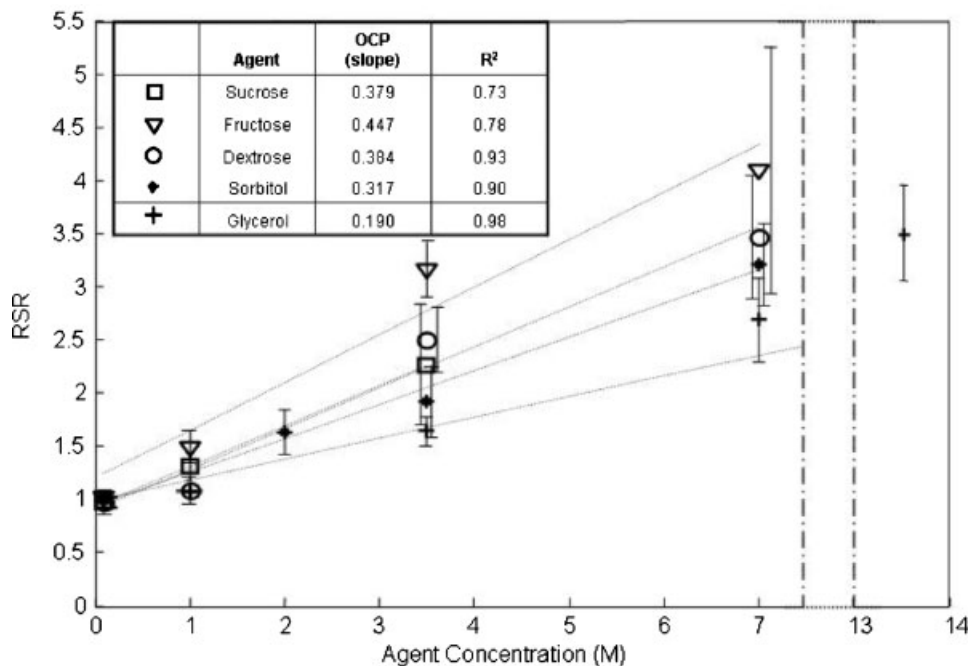


Fig. 3. Reduced scattering ratio (RSR) as a function of agent concentration. Optical clearing potential (OCP) of each agent is defined by slope from linear regression analysis of RSR data and shown in table (inset).

solubility increased with increasing sugar–alcohol chain length [9]. The sugars continued this trend with sucrose having the highest collagen solubility followed by fructose, dextrose, sorbitol, and glycerol. The results presented in Figure 2 are consistent with previously reported measurements of sugar and sugar–alcohol interactions with collagen [13].

Tissue OCP in rodent skin is shown in Figure 3 for the chemical agents. Ratios of tissue reduced scattering coefficient, μ'_s , before and after agent application were quantified as RSR and plotted as a function of agent concentration. Table 1 lists 95% confidence intervals and standard errors for RSR values. The slope from linear regression analysis of RSR data is defined as OCP for each chemical agent. Statistically, the chemicals can be categorized into two distinct groups based on RSR data ($P \leq 0.05$). The sugars and sorbitol were the most effective clearing agents studied with an OCP approximately twice that of glycerol. Glycerol at 13.6 M had a RSR value similar to that of fructose, dextrose, and sorbitol at 7 M. Maximum chemical agent concentrations tested were limited by PBS solubility.

DISCUSSION

Collagen solubility was found to correlate with OCP in rodent skin for sugars and sugar-alcohols. Sucrose, fructose, dextrose, and sorbitol all have higher collagen solubilities than glycerol and exhibit a correspondingly higher OCP ($P \leq 0.05$). Solubility was proposed as a measure of chemical agent interaction with collagen that is necessary for tissue optical clearing [8]. This interaction screens non-covalent attractive forces resulting in inhibition of collagen self-assembly in solution and destabilization of high-order structures in skin. However, our study shows there may be instances in which collagen solubility and OCP do not correlate. Sucrose exhibited the greatest amount of collagen solubility compared to the other chemical agents tested, but had an OCP similar to that of the monosaccharides dextrose and fructose.

Sucrose is a disaccharide composed of the two monosaccharide units dextrose (a.k.a. glucose, six member ring) and fructose (five member ring) bound together by a glycosidic bond (see Fig. 1). We hypothesize that in solution, collagen molecules are free to interact with both monosaccharide units of sucrose, enhancing solubility. In skin, interaction of sucrose with high-order collagen structures is sterically hindered to individual monosaccharide units, resulting in an OCP similar to that of dextrose or fructose.

Previous studies have reported similar relative OCP of agents to those shown herein. For example, 7 M glucose was shown to have the same optical clearing effect as 13 M glycerol as measured by fluorescence through in vitro skin [18]. This is consistent with our study in which collagen solubility predicted glucose to have twice the OCP of glycerol. In addition, it is interesting to note that in efforts to develop non-invasive, optical monitoring of blood glucose levels, a correlation has been reported between a reduction in tissue scattering and glucose concentration [19–21].

TABLE 1. Statistical Analysis of RSR Values Shown in Figure 2

| Agent | 95% Confidence Interval RSR (Standard error) | | | | |
|----------|--|--------------------------------|-------------------------|-------------------------|-------------------------|
| | 0.1 M | 1.0 M | 3.5 M | 7.0 M | 13.6 M |
| Sucrose | 0.9013, 1.0455 (0.0721) | 1.2185, 1.4603 (0.1289) | 1.7571, 2.7651 (0.5750) | | |
| Fructose | 1.0069, 1.0397 (0.0118) | 1.3030, 1.6037 (0.1534) | 2.8999, 3.4201 (0.2654) | 2.9479, 5.2339 (1.143) | |
| Dextrose | 0.8490, 1.1072 (0.1141) | 0.9897, 1.1925 (0.1268) | 2.2021, 2.7975 (0.3077) | 3.1003, 3.8261 (0.3181) | |
| Sorbitol | 0.9013, 1.0089 (0.0549) | 1.1622, 1.4534 (0.1486) at 2 M | 1.7211, 2.2877 (0.2890) | 3.1949, 4.4759 (0.5660) | |
| Glycerol | 0.9434, 1.0290 (0.0379) | 1.0922, 1.1778 (0.0287) | 1.5434, 1.8078 (0.1349) | 2.3095, 3.0837 (0.3951) | 3.0162, 3.9829 (0.4933) |

95% confidence interval and standard error (SE) of the mean RSR value are presented for each agent concentration.

The reduction in tissue scattering was hypothesized to arise from an increase in glucose concentration in local extracellular space, increasing its refractive index to better match that of tissue scatterers. We submit that our results reported here suggest that the molecular interaction of sugars (not limited to glucose) as well as other destabilizing chemical agents with collagen will lead to a reduction in tissue optical scattering.

The correlation between collagen solubility and OCP for sugars and sugar-alcohols suggests that the primary characteristic of effective clearing agents is the ability to screen non-covalent, hydrophilic, intermolecular attractive forces [13] resulting in destabilization of high-order collagen structures. After the initial dissociation of high-order collagen structures into smaller sized light scatterers, other factors such as osmolarity may contribute to the overall increase in skin transparency. The extent to which collagen structures will dissociate is limited by the presence of covalent cross-links [11,12]. We submit that the number and location of these cross-links are determined by the amino acid sequence and hypothesize that because of this, the dissociated collagen structures will have a homogeneous size distribution. We speculate that the hyperosmotic nature of these agents may further contribute by homogenizing inter-scatterer distances. In any case, this and previous studies [9] show that collagen solubility is predictive of skin optical clearing efficacy, in vitro, and indicative of agent-collagen interactions necessary for induced tissue transparency.

A primary physical property has been identified of effective chemical agents for tissue optical clearing. It has been shown that the ability to screen non-covalent intermolecular forces which drive collagen fibrillogenesis correlates with OCP. This property provides a criterion for selection and a basis for the rational design of customized optical clearing agents. Based on the ability of these agents to reduce optical scattering in skin, topically applied optical clearing formulations may be created that enhance light-based therapeutic efficiency, efficacy, and therapeutic outcomes. However, a common property of most optical clearing agents is hydrophilicity which will become prominent as clinical studies are initiated and encounter the challenges posed by the lipophilic stratum corneum barrier of human skin [22,23].

ACKNOWLEDGMENTS

This work was funded by National Science Foundation Faculty Early Career Development (CAREER) Award (A.T.Y.), Arnold and Mabel Beckman Foundation (B.C.), the National Center for Research Resources at the National Institutes of Health (Laser Microbeam and Medical Program, RR01192), and National Institutes of Health (EB004846 to A.T.Y. and AR47551, EB02495, and AR48458, to J.S.N.). J.H. wishes to acknowledge a 2006 ASLMS USAF Travel Grant to present the experimental results described herein at the 26th Annual Meeting of the American Society for Laser Medicine and Surgery in Boston, MA.

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