Molecular interactions of exogenous chemical agents with collagen—implications for tissue optical clearing

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Abstract. Reduction of optical scattering in turbid biological tissues using nonreactive chemical agents has potential applications for light-based diagnostics and therapeutics. Optical clearing effects by exogenous chemical agents, in particular sugars and sugar alcohols, have been found to be temporary with tissue rehydration. Applications with dermatologic laser therapies are now being investigated, but suffer from the inability of studied agents to penetrate the superficial layers of human skin. Selection, design, and refinement of topically effective chemical agents are hindered by a lack of fundamental understanding of tissue clearing mechanisms. We present recent work, particularly from the biochemistry community, detailing molecular interactions between chemical agents and collagen. This body of work demonstrates the perturbative effects of sugars and sugar alcohols on collagen high-order structures at micro- and nanometer length scales by screening noncovalent bonding forces. In addition, these studies emphasize the nonreactive nature of agent-collagen interactions and the ability of noncovalent bonding forces to recover with agent removal and drive reassembly of destabilized collagen structures. A mechanism of tissue optical clearing is proposed based on agent destabilization of high-order collagen structures. © *2006 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.2166381]

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1 Brief Review of Tissue Optical Clearing

The turbidity of most biological tissue limits biomedical applications of light-based diagnostics and therapeutics. Multiple scattering events limit the effective depth over which information about the tissue can be ascertained and the ability to localize embedded features of interest.[1] Optical imaging of deep structures is difficult due to the rapid degradation of image resolution and signal strength with increased depth. Furthermore, optical scattering restricts delivery of a collimated laser beam to subsurface targets such as blood vessels, decreasing the efficacy of light-based therapeutic applications.

Prior studies demonstrate that a reduction in optical scattering can improve the efficacy of light-based techniques in medical applications.[2–10] Nonreactive chemical agents, in particular sugars (e.g., glucose) and sugar alcohols (e.g., glycerol) have been used to temporarily increase tissue transparency.[3–5,11–15] These agents have been investigated primarily in collagenous tissues with initial studies measuring induced increases in light transmittance in ocular sclera.[3] In fact, agents have been shown to be most effective when ap-

plied directly to the mesenchyme of tissue systems such as the dermis of skin. Application of glycerol subdermally *in vitro* or by injection *in vivo* reduced light scattering in skin, improved detection of fluorescence signal[15] from subsurface targets,[5] and enhanced visualization of subsurface blood vessels.[4,11] This optical clearing effect has been studied in other tissue systems such as muscle[12,16] and gastrointestinal tract[13] and was used for agent sensing, where a reduction in tissue scattering occurred with increased blood glucose levels.[2]

Common properties of these chemical agents suggest that refractive index matching and dehydration are possible mechanisms for tissue optical clearing. Light scattering in dermis is predominantly from ubiquitous collagen fibers.[17] Indeed, all studies cited used agents with indices[18,19] of refraction within the range reported for collagen[17,20] (from 1.35 to 1.55). Dehydration is also believed to play a role as all reported agents are hyperosmotic with respect to biological tissue. However, a mechanistic description of tissue optical clearing using refractive index and osmolarity remains to be substantiated experimentally. In fact, there is mounting evidence that these parameters are insufficient to describe tissue optical clearing[15,16,21,22] and have been shown to have no correlation with chemical agent optical clearing potential.[16,22]

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Tissue optical clearing with glycerol shows a strong inverse dependence on the degree of covalent cross-linking present,[21] and *in vitro* studies of collagen gel opacity correlated with sugar and sugar alcohol concentrations[23] suggesting the importance of agent-collagen molecular interactions.

Molecular interactions of sugars and sugar alcohols and their destabilizing and inhibitory effects on collagen structure and fibrillogenesis have been extensively studied within the biochemistry community. Such studies may provide insight into a molecular mechanism of tissue optical clearing and a means of rational selection and design of effective chemical agents. Evidence for a molecular mechanism of tissue optical clearing is presented as well as a review of recent work demonstrating destabilizing and inhibitory effects of sugars and sugar-alcohols on collagen structures and their self-assembly.

2 Clearing Agent Activity on Collagen Reduces Tissue Scattering

Nonlinear optical microscopy (NLOM) is a laser scanning technique that can render thin images from within intact, living tissues.[18,24] Endogenous two-photon fluorescence excitation and second-harmonic generation (SHG) have been characterized for collagen; SHG can provide a unique spectral signature for collagen-specific imaging.[18,19,25] SHG in fibrillar collagen has been used to enhance NLOM image contrast and constituent specific segmentation in skin,[21] cornea,[19] and articular cartilage[25] without using exogenous stains or dyes.

A necessary condition for SHG is that the constituent molecules lack an inversion center. Fibrillar collagens satisfy this condition; its secondary structure is an alpha helix. For type I collagen, two identical $\alpha 1(I)$ and one $\alpha 2(I)$ chains form a triple helix. Despite 30 yr of research, the fine structure and assembly mechanisms of collagen structures remain topics of intense study and inquiry.[26–29] Nevertheless, high-order, macroscopic collagen structures exhibit long-range molecular order, providing a nonlinear medium with characteristic lengths of the order of near-IR wavelengths. Disruption of long-range molecular order extinguishes SHG, making it sensitive to perturbations of collagen structure and useful for optically monitoring denaturation.[30–32]

NLOM was used previously to image collagen with SHG in tissues during optical clearing and subsequent rehydration after applying glycerol and saline, respectively.[21] Tissue systems investigated were fibroblast-seeded collagen tissue constructs and rodent skin, both untreated and fixed. For fibroblast-seeded collagen tissues, application of glycerol resulted in loss of SHG from collagen and concomitant increase in gel transparency. Loss of SHG was indicative of collagen destabilization by glycerol, an effect demonstrated with electron[33] and polarization light microscopy[21] in glycerinated rodent tail tendon. Application of glycerol to rodent skin did not extinguish SHG signal, but fibrous collagen morphology unraveled to a matted appearance concomitant with increased tissue transparency.

Subsequent rehydration with saline in fibroblast-seeded collagen tissues restored SHG signal, fibrous collagen morphology, and gel turbidity. Similarly, rehydration of rodent skin with saline resulted in reformation of fibrous collagen morphology and return in tissue turbidity. Differences in collagen structure between fibroblast-seeded collagen tissue and rodent skin following glycerol application, as observed by NLOM, were hypothesized to be due to the presence of native covalent cross-links. Indeed, the introduction of an abundance of covalent bonds through formalin fixation defeated glycerol induced optical clearing and structural changes to collagen as measured by NLOM.

3 Destabilizing Effect of Glycerol on Collagen in Rodent Tail Tendon

The destabilization of native collagen structures using nonreactive chemical agents suggests the primary bonding forces for these high order protein assemblies are noncovalent in nature. Type I collagen is the predominant structural component in most biological tissues and shows increased thermal stability against denaturation in solution with sugar alcohols.[34–36] The earliest evidence of native collagen dissociation using glycerol was reported over 20 yr ago using transmission electron microscopy (TEM) and x-ray diffraction (XRD) techniques to measure collagen fiber ultrastructure in rodent tail tendon following one of six exposures: (1) water, (2) phosphate buffer, (3) glutaraldehyde, (4) glutaraldehyde followed by glycerol, (5) glycerol, or (6) glycerol followed by phosphate buffer.[33] Consistent with observations[21] using NLOM, only glycerol treatment induced swelling of interfibrillar space and dissociation of collagen fibrils into microfibrils (and loss of characteristic banding in some regions), as observed using TEM and molecular disorder as measured with XRD. Ultrastructurally, glutaraldehyde fixation (exposure 4) nullified, whereas rehydration with phosphate buffer (exposure 6) reversed the dissociative effects of glycerol.[33] These ultrastructural and NLOM studies show that (1) bonding forces for high-order collagen structures are primarily noncovalent in nature; (2) glycerol interrupts these bonding forces resulting in disassembly; and (3) on rehydration, these bonding forces are restored resulting in reassembly of high-order collagen structures.

4 Molecular Interactions of Agents with Collagen *In Vitro*

Studies using synthetic collagen-like peptides show that stable formation of secondary, tertiary and, consequently, higher order structures depend on specific binding sites.[37] Stability of secondary and tertiary structures was shown to be enhanced by inductive (electronegativity) effects provided by hydroxylated proline residues.[38] For quaternary and higher order structures, attractive forces mediated by hydrophilic environments[39] are preeminent, and sugars and sugar alcohols have been used effectively to elucidate collagen interactions and self-assembly mechanisms.

4.1 *Hydrogen Bonding in Fibrillogenesis*

The dynamics of collagen fibril formation has been described as nucleation followed by growth during which higher order structures (fibrils) develop.[23] Noncovalent forces driving collagen fibrillogenesis could include, for example, hydrogen bonding, van der Waals forces, and steric interactions. Sugars and sugar alcohols have been used to modulate and characterize these forces[23,40–44] in conjunction with measurement techniques utilizing osmotic pressure to study macromolecular interactions.[45] Typically, these techniques apply force through
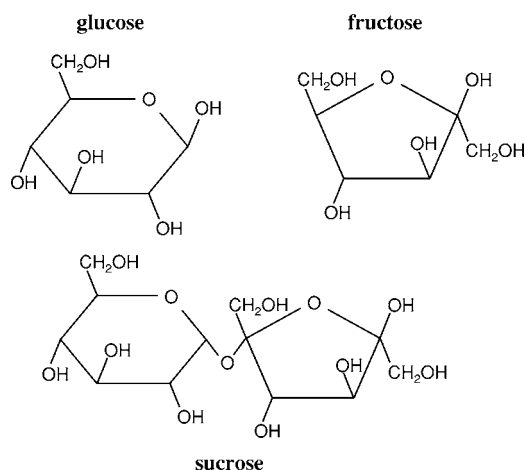
Fig. 1 Chemical structure representations of sugars glucose, fructose, and sucrose.

Fig. 2 Chemical structure representations of sugar alcohols ethylene glycol, glycerol, and sorbitol and 1,2- and 1,3-propane diol.

osmotic pressure via polymer solution and measure intermolecular distance by XRD. Force-distance measurements have been performed using concentrated collagen thin films immersed in solutions of polyethylene glycol[40–43,45] (PEG). PEG does not penetrate these thin films, thus exerting osmotic pressure on the collagen fibers in a manner directly related to its concentration. Osmotic removal of water decreases the molecular (interaxial) separation of triple helices; this osmotic force is balanced by repulsion. Decreasing PEG concentration was shown to correspond with decreasing osmotic pressure and increasing interaxial distances until attractive forces dominated. At this point, decreasing applied osmotic pressure did not result in further increases in interaxial distances due to intermolecular interaction forces. These attractive forces are responsible for molecular recognition and drive collagen fibrillogenesis.

Collagen attractive forces may be characterized with the addition of sugars and sugar alcohols. These agents screen collagen attractive forces resulting in force-distance measurement curves that reflect intermolecular repulsion (i.e., interaxial distance continued to increase with decreasing osmotic pressure). Collagen attractive forces were characterized by taking the difference between curves, with and without agents, as a function of interaxial distance.[40–43]

Force-distance measurements of collagen led to two significant findings in determining the mechanism for fibrillogenesis: (1) the repulsive force increases exponentially with a decrease in interaxial spacing (1.5 to 2.0 nm), and (2) both interhelical spacing and net repulsive force decrease with an increase in temperature.[42] These findings, along with the discovery that force-decay lengths and force magnitudes were insensitive to ionic strength at high osmotic stress, suggest that either "hydration forces" between polar surfaces or the "hydrophobic effect" of nonpolar moieties is the dominant force in fibrillogenesis. Although the temperature sensitivity of attraction is qualitatively consistent with both hydration forces and hydrophobicity, the magnitude of this sensitivity suggests a hydrophilic mechanism.[43]

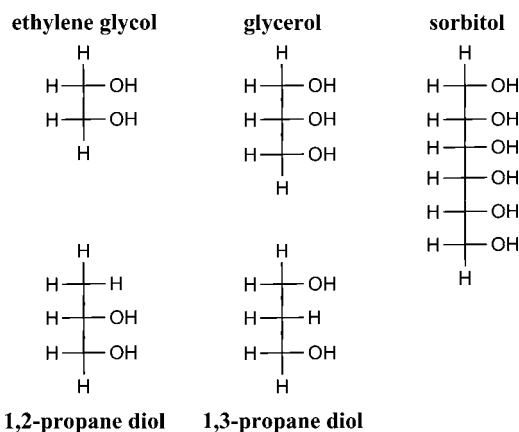A mechanism for fibrillogenesis was further defined when considering the effects of pH on collagen fiber formation. Collagen fibrillogenesis was strongly favored at physiologic pH ($\sim$7.4). However, when pH was lowered to 6, there was substantial weakening of the attraction between collagen helices—consistent with titration of specific hydrophilic residues.[43] pH effects suggest involvement of histidine residues which have the lowest pK$_a$ value of the three basic amino acids making it the most sensitive to reduction of pH from 7.4 to 6. At neutral pH, histidine is neutral and forms hydrogen bonds as a proton acceptor. Reducing pH protonates histidine, whereby it becomes a hydrogen bond donor and disrupts the interactions between helices. The reaction of collagen to pH, in conjunction with force-distance measurements, support hydrophilic interactions as the main driving force for fibrillogenesis.[21,40,42,43]

4.2 Collagen Solubility (Inhibition of Fibrillogenesis)

Addition of sugars and sugar alcohols was shown to enhance collagen solubility in buffered saline by masking hydration mediated attractive forces between triple helices and inhibiting fibrillogenesis.[23,36,40–44] These chemical agents slow and limit fibrillogenesis by disrupting hydrogen bond facilitated water bridges between collagen triple helices. The efficiency of fibrillogenesis in the presence of glucose, fructose, and sucrose (see Fig. 1) as well as ethylene glycol, glycerol, and sorbitol (see Fig. 2) has been studied previously and showed an inverse dependence on agent chain length.[23,40] Ethylene glycol was the shortest (two-carbon chain) agent tested and displayed little effect on collagen fibrillogenesis.[40] Inhibitory effects of glycerol on collagen self-assembly are well documented.[23,40,44] Sorbitol is twice as long as glycerol (six and three carbons, respectively) and was shown to have twice the collagen solubility.[40] Glucose and fructose are six-carbon sugars and was shown to have greatest collagen solubility when compared to sugar alcohols[40] (ethylene glycol, glycerol, and sorbitol). Sucrose, a disaccharide of glucose and fructose, showed similar inhibitory effects on collagen fibrillogenesis as with the monosaccharides.[23]

Insight into the difference in collagen solubility between ethylene glycol and glycerol can be gained by comparison studies of 1,2- and 1,3-propane diol, which both have similar dielectric properties, e.g., their indices of refraction are 1.43 and 1.44, respectively. If electrostatic, van der Waals, or hydrophobic interactions were the major attractive forces in fi-

brillogenesis, these two agents should have similar collagen solubilities. In fact, 1,3-propane diol had ~20 times greater collagen solubility (comparable with glycerol) than 1,2-propane diol which showed comparable collagen solubility with ethylene glycol.[40]

Collagen solubility of propane diols, ethylene glycol, glycerol, and sorbitol suggests a stereochemical effect.[40,43] For propane diols, adjacent or terminal placement of hydroxyl groups on the three-carbon backbone has dramatic effects on collagen solubility. This is reflected in sugar alcohols where ethylene glycol (glycerol) and 1,2- (1,3-)propane diol have similar distances between hydroxyl end groups. Placement of hydroxyl end groups on 1,3-propane diol and glycerol may correspond to the spacing of hydrophilic interaction sites of collagen tertiary structures. The fact that sorbitol has twice the collagen solubility as glycerol is supportive, but studies with sugar alcohols of other chain lengths would further refine this hypothesis.

5 Rational Selection and Design of Tissue Optical Clearing Agents

Evidence of a molecular mechanism for tissue optical clearing was presented, providing insight for rational selection and design of exogenous, nonreactive clearing agents. For glycerol, it has been established that the induced increase in tissue transparency results from the destabilization of high-order collagen structures.[21,33] This destabilization effect was shown to be reversible structurally[33] and mechanically.[46] Glycerol also has been shown to have a stabilization effect on collagen tertiary structure against thermal denaturation.[34,36] This apparent dichotomy may be explained by attractive forces involved in the collagen protein structure hierarchy.

Synthetic collagen-like peptides have been used to demonstrate that self-assembly of collagen I secondary and tertiary structures is driven by specific hydrophobic and electrostatic binding sites[37] and that stability of these structures is enhanced by inductive (electronegativity) effects conferred by hydroxyproline residues.[38] For higher order structures, osmotic stress and x-ray scattering measurements using sugars and sugar alcohols demonstrated that hydrogen bonding is the primary bonding force between collagen triple helices.[40–43] Screening of hydrogen bonding forces by these chemical agents was shown to destabilize/disassociate high-order collagen structures and results in increased tissue transparency.[21,23,33] Comparison studies of collagen solubility in clearing agents suggest a testing protocol to determine dose and evaluate potential optical clearing efficacy in vitro prior to testing *in vivo*.

Clearing agent interactions with collagen are nonreactive. In addition to tissue optical properties, however, clearing agent interactions with collagen, the primary structural protein in most tissues, suggest other properties may be affected as well, for example, tissue mechanical properties. Mechanical testing (stress-strain measurements) of collagenous tissues reveals a bimodal behavior.[47] These measurements are typically acquired relative to a reference state, which is attained after preconditioning tissue to low stress. For low strain, collagenous tissues show compliance, accommodating stretch by collagen fiber reorientation and undulation straightening. Tissue stiffening follows when these degrees of freedom are ex-

hausted and stretch is accommodated by matrix proteins, in particular, collagen. The compliant and stiff response of tissue can be associated with noncovalent and covalent bonding forces, respectively.[47]

The effects of glycerol on the mechanical properties of epicardium and their reversibility have been characterized using stress-strain measurements.[46] Bovine epicardium was used as a model 2-D collagenous tissue. Following glycerol application, "cleared" epicardium exhibited bimodal mechanical responses with a reduced compliant region. This reduction in the compliant region was consistent with glycerol screening of noncovalent interactions. On rehydration with buffered saline, epicardial mechanical response was completely restored to pre-glycerol-treated levels.[46] These results were encouraging in that the mechanical integrity of treated collagenous tissue was not permanently compromised by optical clearing but, in fact, was completely reversible.

Agent-induced destabilization of collagen structures leading to reduced optical scattering in tissue was shown to be reversible, but hindered by covalent cross-linking.[21,33] This suggests the use of clearing agents adjuvant with clinical light-based diagnostics and therapeutics may be limited by factors such as patient age. In addition, current challenges to tissue optical clearing include epidermal penetration by clearing agents. Effective, topically applied clearing agents require properties that facilitate breach of the lipid barrier yet retain hydrophilic properties to destabilize collagen structures. Synergistic approaches have been investigated, including topical applications of miscible solutions of transepidermal carrier and clearing agents.[9,10,14] Solutions of dimethyl sulfoxide and glycerol applied topically to gastric tissue was shown to enhance light transmission when compared to administering glycerol alone.[14] The clinical utility of topical lipophilic polypropylene glycol and polyethylene-glycol-based polymer mixtures has been demonstrated in laser removal of tattoos, showing reduced skin injury and increased therapeutic efficacy when compared with using laser treatment alone.[9] These results are promising and warrant further exploration of topical clearing regimens.

Studies characterizing the effects of clearing agents on tissue properties and function continue; initial results suggest modulation of tissue physical properties are temporary and completely reversible.[3,4,46] Observed agent effects on microvasculature and blood flow[11,48] suggest biological response may be elicited, which may impact tissue function. Comparative studies with accepted treatment protocols are required to further define efficacy enhancement for light-based therapeutics and to characterize potential side effects.

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