

Reversible Dissociation of Collagen in Tissues

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The turbidity of most biologic tissues hinders the use of lasers for diagnostic and therapeutic purposes. Hyperosmotic agents such as glycerol have been used to alter the optical scattering properties of turbid tissues. The mechanism of this effect, “optical clearing”, however, remains incompletely understood. Multiphoton microscopy utilizing second harmonic generation can be used to monitor collagen structural changes in the presence of glycerol. This study suggests that the use of glycerol for tissue “optical clearing” screens noncovalent intermolecular interactions between low-order collagen structures, resulting in fiber disassembly.

Dissociation of collagen fibers in native and engineered tissues in the presence of glycerol and reassociation with the application of saline are imaged dynamically. Collagen fiber reassembly is governed by the displacement of glycerol by saline in native and engineered skin. These results reveal the assembly process of high-order collagen structures and propose a molecular mechanism for the increase in tissue transparency observed after glycerol application. Key words: extracellular matrix/glycerol/multiphoton microscopy/tissue engineering. *J Invest Dermatol* 121:1332–1335, 2003

The biomedical use of lasers for therapeutic and diagnostic purposes is hindered by the turbidity of most biologic tissues. The technical challenge of optical imaging in highly scattering media has led to the introduction of novel techniques such as two-photon microscopy (Denk *et al*, 1990) and optical coherence tomography (Huang *et al*, 1991). Another approach under investigation is the reduction of tissue optical scattering by applying hyperosmotic agents (Tuchin *et al*, 1997) such as glycerol (Vargas *et al*, 1999; Choi *et al*, 2002). The mechanism of “optical clearing”, however, remains incompletely understood.

The ubiquity of collagen in tissues has led to it being one of the most widely studied biologic molecules (Prockop and Kivirikko, 1995). Collagen has a hierarchical structure in which the tertiary structure is a triple helix. The quaternary structure is represented by fibrils that assemble into macroscale collagen fibers that provide the architecture for tissues, including human skin and ocular sclera.

Collagen has an appreciable nonlinear susceptibility for second harmonic generation (SHG) (Fine and Hansen, 1971). The helix of the collagen secondary structure is noncentrosymmetric, satisfying a condition for SHG, which self-assembles into higher order structures. The assembly of quaternary fibrils into fibers provides an ordered nonlinear medium with a cross-sectional path length comparable to near infrared wavelengths ($\lambda = 800\text{--}1500$ nm). SHG has been used in multiphoton microscopy (MPM) for specific optical imaging of collagen in tissues (Guo *et al*, 1997; Moreaux *et al*, 2000; Campagnola *et al*, 2001; Williams *et al*, 2001; Stoller *et al*, 2002a; Yeh *et al*, 2002). Collagen has been shown to

have a dominant uniaxial second-order nonlinear susceptibility component aligned along the fiber axis (Stoller *et al*, 2002b). The SHG signal is strongly dependent on the material order, making it highly sensitive to structural changes.

When a hyperosmotic agent such as glycerol is applied to skin, an increase in transparency is observed (Vargas *et al*, 1999, 2001). This “optical clearing” effect can have profound implications for improved laser-based diagnostics and therapeutics. The purported mechanism for “optical clearing” is a decrease in refractive index mismatch between collagen and the surrounding dermal medium due to a combination of local dehydration and partial replacement of water by glycerol (Vargas *et al*, 1999, 2001). The work presented herein provides the framework to study the association mechanisms of high-order collagen structures as well as the effects of hyperosmotic agents in native and engineered skin. This research project has been approved by the Institutional Review Board (IRB).

MATERIALS AND METHODS

MPM The MPM imaging system has been described previously (Agarwal *et al*, 2001). A Ti:Al₂O₃ oscillator (Coherent, Santa Clara, CA) pumped by a frequency doubled Nd:YVO₄ solid state laser (Coherent) generates 800 nm, 150 fs pulses at a repetition rate of 76 MHz. The laser is coupled into an inverted microscope (Zeiss, Jena, Germany) via two galvanometer driven mirrors (Cambridge Technology, Cambridge, MA) to facilitate laser scanning in the focusing plane. Imaging signals are collected in the backscattering geometry by the focusing objective and are directed to a photomultiplier tube (Hamamatsu Photonics, Hamamatsu City, Japan) for photon counting and intensity image rendering. To isolate the SHG signal from collagen, a bandpass filter at 400 nm (CVI, Albuquerque, NM) was placed in front of the photomultiplier tube. Image integration time was approximately 40 s.

Polarized light microscopy (PLM) Tissue samples were mounted between two glass slides for viewing on an inverted microscope (Olympus, Melville, NY). Linear polarizers (CVI) were placed above the condenser and sample. Their polarization axes were adjusted to extinguish background light. Digital photographs were recorded with a color CCD

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Abbreviations: SHG, second harmonic generation; MPM, multiphoton microscopy; PLM, polarized light microscopy; RTT, rodent tail tendon.

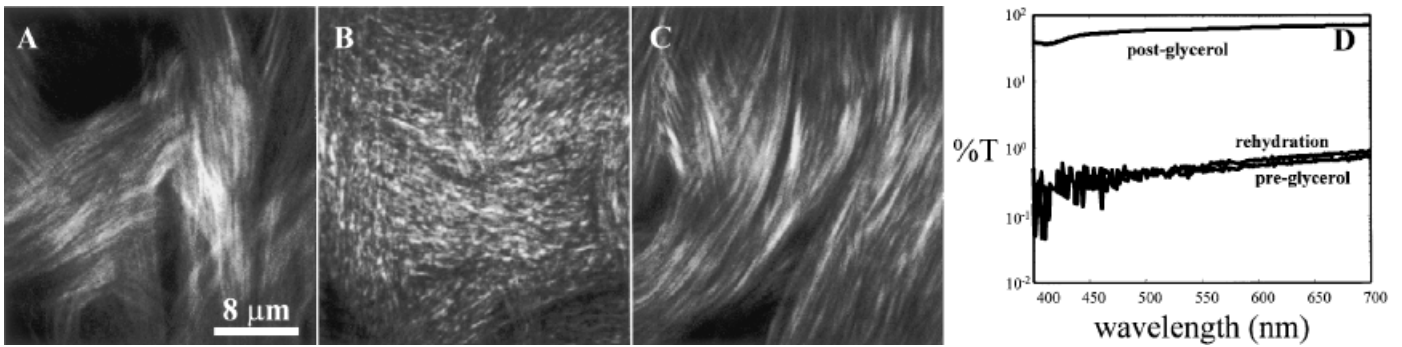


Figure 1. Reversible effect of glycerol on rodent dermis. (A) MPM prior to glycerol application. (B) MPM after glycerol application. (C) MPM after rehydration with PBS. (D) Transmission spectra of rodent dermis pre- and post-glycerol application and following rehydration with PBS.

camera (Olympus) mounted on the microscope. Each image contained 1600×1200 pixels in 24 bit color. To maximize image contrast, exposure times ranged between 2 and 70 ms.

Native tissues Rodent skin specimens were obtained from the scalps of adult animals (400 g, Sprague Dawley). The subcutaneous layer was dissected surgically. The skin was treated with dispase (Invitrogen, Carlsbad, CA) to remove the epidermis.

Tendons were surgically removed from rodent tails immediately following sacrifice. The tail was excised from the body, skin was removed, and tendons were dissected free. The excised tendons were rinsed sequentially in phosphate-buffered saline (PBS) (Invitrogen) and then 70% ethanol and stored in PBS at 4°C . Prior to experiments, tendon temperature was allowed to equilibrate with ambient conditions.

Engineered tissue model (raft) Construction of the raft model has been described previously (Agarwal *et al.*, 2001). Solubilized rodent tail type I collagen (≈ 3 mg per ml) (BD Biosciences, Bedford, MA) was brought to physiologic pH with normal human dermal fibroblast cells (10^5 cells per ml). The fibroblast cells were harvested from human foreskin using an approved protocol (HS# 2001-1727, Institutional Review Board, University of California, Irvine, CA). The data presented were obtained from rafts harvested after 5 d.

Reversible clearing measurements For dynamic MPM imaging, turbid tissues were placed on a coverslip and imaged using an inverted microscope. The laser was focused from below approximately $30 \mu\text{m}$ into the center of the sample. Glycerol (Sigma-Aldrich, St Louis, MO) and PBS were applied from above over the field of view. For tissue "optical clearing", five drops of 13 M glycerol were applied and MPM images were recorded every minute. Typical time for "optical clearing" was 20 min as observed by the investigators. To reverse the clearing effect, excess glycerol was removed and five drops of PBS were applied over the field of view. MPM images were recorded every minute and continued beyond the return of tissue turbidity.

For PLM, rodent tail tendon (RTT) was placed between two microscope slides for imaging. The tendon was then placed in a Petri dish of glycerol to induce "optical clearing" for approximately 5 min. Excess glycerol was removed and the tendon was replaced between two slides for imaging. To reverse the clearing effect, the tendon was placed in a Petri dish of PBS. Turbidity returned in approximately 5 min. Representative images are presented.

RESULTS AND DISCUSSION

Images of rodent dermis in the presence of 13 M glycerol and upon subsequent rehydration with PBS are shown in **Fig 1(A–C)** using MPM. Fibrous dermal collagen prior to glycerol application is seen in **Fig 1(A)**. Following glycerol application, the dermis becomes optically transparent and the fibrous structure unravels into a matted morphology (**Fig 1B**). Removal of excess glycerol and subsequent application of PBS lead to a recovery of the collagen fibrous structure (**Fig 1C**). An ultraviolet–visible spectrophotometer was used to measure an increase in transmission of two orders of magnitude in the "cleared" sample (400–700

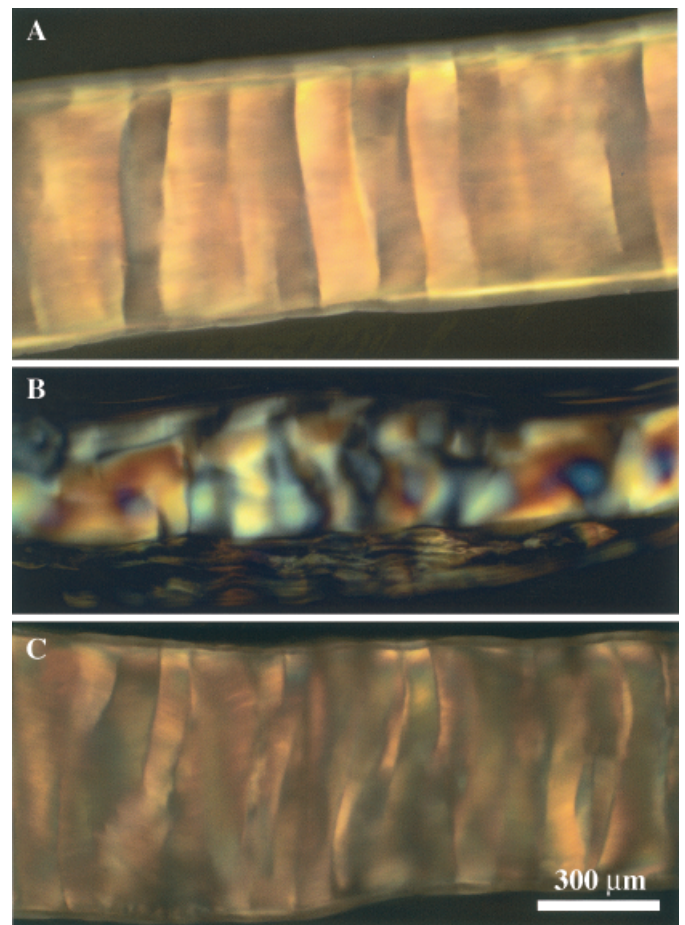


Figure 2. Reversible loss of collagen fibril organization in RTT following glycerol application observed by PLM. (A) Banding patterns observed in RTT using PLM indicates ordered fibril organization. (B) Loss of banding patterns following glycerol application suggests loss of fibril organization. (C) Rehydration of RTT.

nm, **Fig 1D**). Following PBS application, tissue turbidity returned as observed by the investigators and measured by light transmission spectra shown in **Fig 1(D)**.

Glycerol has a strong affinity for collagen and acts to preserve the triple helical structure from thermal denaturation (Na, 1986). The association of glycerol to collagen molecules inhibits fibrillogenesis by preventing intermolecular interactions and has been used to study fibril self-assembly dynamics *in vitro* (Na *et al.*, 1986). Our measurements *in situ* on native dermis are suggestive that

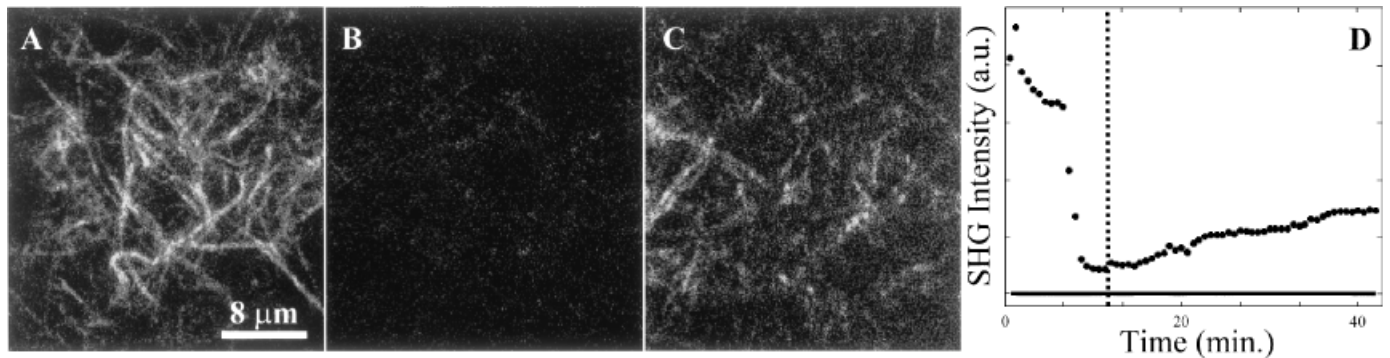


Figure 3. Reversible effect of glycerol on organotypic raft tissue. (A) MPM image of the raft. (B) Disappearance of SHG in collagen following glycerol application. (C) Partial recovery of SHG indicative of re-formation of collagen fibers. (D) Representative time dynamics of SHG signal loss and recovery in the raft due to glycerol and subsequent rehydration using PBS indicated by the vertical line. Each data point represents approximately 40 s.

screening of noncovalent intermolecular interactions by glycerol leads to a loss of fibril organization or the dissociation of collagen fibers (Fig 1B). Cross-linking in native collagen (Tanzer, 1973) maintains molecular order for SHG, preserving the SHG signal (Fig 1B).

The dissociative effects of glycerol can be observed on a longer length scale using PLM. PLM can be used to measure collagen birefringence, qualitatively and quantitatively, which is indicative of molecular structure and macroscopic organization (Wolman and Kasten, 1986). Figure 2 shows PLM images of RTT and the effects of glycerol application and subsequent rehydration with PBS. The dark background in each of the images demonstrates the extinction of the crossed polarizers. Characteristic banding patterns in RTT as seen in Fig 2(A) are a result of macromolecular, ordered assembly of collagen. Following glycerol application, the banding pattern is no longer evident (Fig 2B) and the width of RTT is decreased, presumably from dehydration by the hyperosmotic effect of glycerol. Color variations are suggestive of local path length differences in RTT (Wolman and Kasten, 1986). The loss of the banding pattern in Fig 2(B) indicates loss of long range order and suggests fiber dissociation (Usha and Ramasami, 2002). Upon rehydration with PBS, the banding patterns returned, as seen in Fig 2(C), indicative of a return of collagen fibril organization.

Engineered (raft) tissue models were constructed with human dermal fibroblast cells and reconstituted rodent tail type I collagen (Agarwal *et al*, 2001). Collagen fibers (Fig 3A) self-assemble at physiologic pH from acidic conditions. As such, they lack the cross-linking found in native collagen (Gross, 1964), which could modulate the effects of glycerol on microstructure organization. Images of the raft after 5 d in culture are shown in Fig 3 using MPM. Following glycerol application, intermolecular interactions that lead to fibrillogenesis are shielded, resulting in dissociation of collagen fibers and loss of SHG (Fig 3B). Qualitatively, raft transparency following glycerol application was considerably greater than that observed in rodent skin. Displacement of glycerol from collagen molecules by PBS results in reassembly of collagen fibers and a return of SHG (Fig 3C). Dynamics of collagen dissociation caused by glycerol and subsequent fiber reassembly with PBS can be monitored by analysis of time-resolved SHG intensity images (Fig 3D). Shown in Fig 3(D) is a representative time trace of four experiments. The integration time for each data point is approximately 40 s and represents the average of the maximum value in each column of the intensity images. Upon glycerol application, the SHG intensity falls to a minimum due to loss of molecular order in the collagen fibers. Upon PBS application (vertical line) and displacement of glycerol from collagen molecules, the SHG intensity rises concomitantly with reassembly of collagen fibers. Fiber reassembly is governed by the extent of glycerol displacement from the collagen surface, which is driven by local concentration gradients. Complete

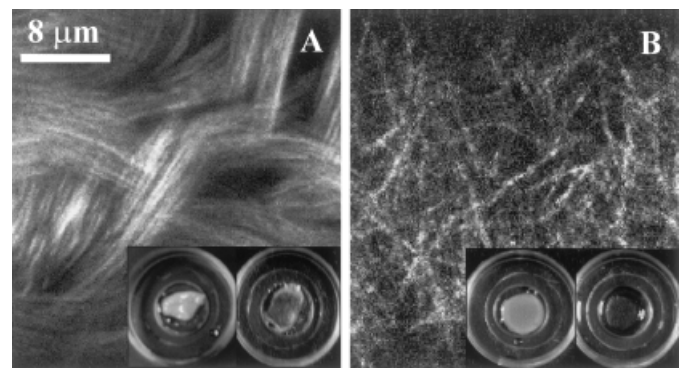


Figure 4. Extensive cross-linking induced by formaldehyde fixation inhibits collagen dissociation and optical clearing by glycerol. Preservation of collagen fiber structure as shown by the MPM image of formalin-fixed tissue following glycerol application to rodent dermis (A) and raft tissue (B). Shown in the inset are digital photographs of fixed and non-fixed dermis (A) and raft tissue (B) following glycerol application.

displacement of glycerol should lead to full recovery in SHG intensity.

Extensive cross-linking induced by 4% formaldehyde solution (Fox *et al*, 1985) can inhibit the dissociation of collagen and “optical clearing” induced by glycerol. Shown in Fig 4 are MPM images of fixed rat dermis (A) and raft tissue (B) following glycerol application. The collagen morphology of fixed rat dermis observed in Fig 4(A) is similar to the morphology observed in neat dermis (Fig 1A). Similarly, the collagen morphology of fixed raft tissue (Fig 4B) is comparable to neat raft tissue (Fig 3A). Due to extensive cross-linking following fixation, glycerol is unable to “clear” the tissues. Shown in the inset of Fig 4 are photographic pictures comparing fixed and nonfixed tissues following glycerol application.

Our measurements suggest a reversible dissociative effect by glycerol on collagen, which can have implications on the optical scattering properties of tissues. Glycerol has been used as an “optical clearing” agent with the rationale of refractive index matching between the agent and collagen (Vargas *et al*, 1999, 2001). Previous work supports the assumption that collagen fibers act as the primary light scatterers in skin (Saidi *et al*, 1995). Our results suggest a molecular mechanism for “optical clearing” due to a loss of order in fibril organization following glycerol application in rodent dermis and raft tissue. The change in collagen structure and size can lead to a substantial reduction in tissue light scattering. Our measurements also suggest that the extent of disorder and the final distribution of particle sizes are limited by the presence of covalent cross-links. Future studies are planned to use spectroscopic and goniometric methods for determining skin

scattering properties during glycerol application and correlating these measurements with MPM images.

In addition to providing the structural framework for biologic tissues, collagen serves a plurality of functions. Many of these functions can only be realized when collagen has self-assembled to its high-order macroscopic structure. Dynamic imaging using MPM *in situ* has shown that, even with cross-linking found in native tissues, the stability of these structures is easily perturbed by exogenous agents (Figs 1, 3), altering the optical properties of the tissue. The sensitivity of MPM to collagen structure makes it a useful modality to study collagen-related diseases and dystrophies *in situ*.

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