Characterization of Ultrastructure of Collagen Hemostats and their Relationship to Healing

Marc Long, PhD1, Jessica Stevens1, Doug Keene2, Hans Peter Bachinger3, James San Antonio1.

1Stryker Orthobiologics, Malvern, PA, USA, 2Shriner's Hospital for Children, Portland, OR, USA.

Disclosures:

M. Long: 3A; Stryker. 4; Stryker. J. Stevens: 3A; Stryker. D. Keene: 6; Stryker. H. Bachinger: 6; Stryker. J. San Antonio: 3A; Stryker.

Introduction: Collagen is a versatile biomaterial and has been widely used in many medical applications including hemostasis where it creates a scaffold for the clot to form and stop bleeding [2]. How the collagen is further remodeled by the body depends on the nature and conformational state of the collagen when it is applied to the bleeding site. Pepsinized collagen is a unique microfibrillar collagen produced when the enzyme pepsin is used to release the collagen from hide tissue. Pepsin clips away the telopeptide ends of the collagen molecule rendering the collagen markedly less immunogenic to the human body [3]. Furthermore, the pepsinization-based process has been shown to produce microfibrillar collagen as close as possible to its natural state [4], which may be beneficial for the remodeling of the collagen during hemostasis towards facilitating healing in addition to stopping bleeding. The purpose of this evaluation was to characterize the ultrastructure of collagens used in hemostatic products in various forms (soluble or insoluble/fibrous) and assess whether they can be characterized as microfibrillar and exhibit the associated conformational flexibility to transition to the fibrillar state.

Methods: Pepsinized collagen (PC) solutions (N=10) were obtained from commercial GMP lots and dissolved at 3 mg/ml in 0.013N HCl. Fibrillar collagens were commercially available as hemostasis products: Instat/Ethicon (FCI) and Avitene/CR Bard (FCA). For acid solubility testing, FCI and FCA samples (N=9 for each) were suspended at 4 mg/ml in 0.013N HCl, in 15 ml centrifuge tubes, vortexed for 5 sec and agitated gently on an orbital shaker for 48 hr at ambient temperature. Samples were qualitatively assessed for solubility by holding the sample tubes against a dark background and visually inspecting for turbidity or suspended material. PC solutions were precipitated in various ways to prepare fibrillar suspensions for TEM (Transmission Electron Microscopy) analysis on a FEI Tecnai G2 microscope operated at 120KV. To generate microfibrillar collagen, the collagen solution was dialyzed into 0.14M NaCl, 0.2M NaHPO4 buffer, pH 7.3, at 4°C for 3 days. Aliquots (3 µL) of 1:10 dilutions of PC fibril suspensions were pipetted onto a carbon-coated grid, then were stained by adding a drop of 3.0% phosphotungstic acid, pH 7.3 and dried prior to TEM examination. To prepare collagens in forms ranging from microfibrils to mature-native type fibrils, the collagen solution was either precipitated with 0.2M NaHPO4 buffer, pH 11.2, at room temperature for 3 hr, or was clarified by centrifugation at 10,000 RPM for 75 min at 14°C, and precipitated with phosphate buffered saline (PBS), pH 7.2 (Gibco, Life Technologies) at 30°C for 2.5 hr. FCI and FCA samples were observed to be acid-insoluble and fibrous and thus did not require phosphate precipitation. Therefore, these samples were either suspended at 2.4 mg/mL in 0.14M NaCl, 0.2M NaHPO4 buffer, pH 7.3 or at 8.0 mg/ml in PBS, pH 6.9. For TEM examination, suspensions of phosphate-precipitated PC, FCI or FCA samples were pelleted in eppendorf tubes, fixed in 3.0% glutaraldehyde containing 0.01% tannic acid, osmicated, dehydrated to 100% ethanol, washed with propylene oxide and embedded in Spurrs epoxy. Sections (80nm) were cut with an ultramicrotome and mounted on formvar-coated grids, followed by staining in uranyl acetate and lead citrate. For each sample type and processing condition, five to fifteen electron micrographs were generated. For some FCI and FCA samples, the MT XR41 side-entry digital camera software on the electron microscope was used for morphometric analysis of collagen fibril diameters within micrographs. For microfibril and fibril diameter measurements of PC fibrils, TEM images were magnified using the Ricoh MPC 5000 photocopier and fibrils from three electron micrographs were measured with a ruler and their diameters extrapolated from the magnifications provided on the micrographs.

Results: PC solutions were acid soluble, based on their translucent appearance, which is characteristic of pure atelopeptic collagen with a relatively low content of intermolecular crosslinks. Neither FCI nor FCA samples were acid soluble, as evidenced by their turbid appearance. This behavior is characteristic of moderately to highly cross-linked, acid-insoluble collagenous materials. Precipitated PC solutions appeared as a population of randomly-oriented thin fibrils of diameters < 30 nm (Fig. 1). Such collagen fibrils are considered “microfibrillar”, which was defined as comprising a population where the majority of fibrils (> 80%) have diameters of < 30 nm [4]. Morphometric analysis was performed, confirming PC to be microfibrillar and indicating the average microfibril diameter to be ≈ 13 nm (Fig. 4). Fibrillar PC preparations contained some microfibrillar collagen as well as loose rope- or ribbon-like bundles of microfibrils or diffuse fibrils with indistinct cross-fibril striations (Fig. 2). After epoxy embedding and sectioning, fibril-like structures were evident, some of which exhibited periodicity (Fig. 3). These data suggest that PC formed microfibrils which in some cases aggregated into larger, incompletely formed native-type fibrils. Based on these observations, some PC was also PBS-precipitated at 30°C to yield mature (fully formed) native-type collagen fibrils exhibiting the hallmark ≈ 67 nm molecular periodicity and having average diameters of ≈ 90 nm (Fig. 4). These findings confirmed previous
work using TEM and DSC that revealed pepsinized collagen to form native-type fibrils of both banded and non-banded morphologies [4]. These observations highlight the conformational versatility of PC as it will form classic microfibrils, or intermediate or mature native-type fibrils according to the conditions to which it is exposed. In contrast, TEM inspection of FCI and FCA samples both to be comprised of thick collagen fibrils or more commonly, fibril bundles. Many fibrils displayed molecular periodicities consistent with native-type or native fibrils. Diameters of fibrils in each preparation were measured, showing broad distributions ranging from $\approx 35-300$ nm for FCI and $\approx 170-280$ nm for FCA. Notably, not a single fibril from either preparation exhibited a diameter narrow enough to be considered microfibrillar (Fig. 4).

**Discussion:** The solubility and ultrastructural characteristics of FCI and FCA suggest these materials to be comprised of moderately to highly crosslinked native or native-type collagen fibrils, and lack classical collagen microfibrils. Moreover, unlike PC, FCI and FCA do not have the conformational flexibility to enable their conversion between the microfibrillar and fibrillar states according to the ambient conditions. These results imply that when used *in vivo*, pepsinized microfibrillar collagen has the potential to rapidly transition between multiple conformational forms with potential significance to the facilitation of healing of application sites.

**Significance:** The collagen components of hemostats are potent platelet aggregating and activation factors, yet they also may support the regeneration of the injured tissue associated with the blood clot[1]. Collagen function during hemostasis depends on its molecular structure, which in turn affects their conformational flexibility and structural role during healing.

**Acknowledgments:** N/A

**References:** 1. Raher, Wound Care Institute Newsletter, 5 (1999); 2. Achneck et al., Annals of Surgery, 251 (2010); 3. Lynn et al., J
Figure 1. Representative TEM micrograph of bovine collagen solution containing phosphate precipitated at room temperature (8.5-9.0). The majority of the collagen comprises loosely wound aggregates or fibrils with some evidence of periodicity; i.e., having a ladder appearance. The collagen microfibrils making up these structures existed as single micro fibrils that are also apparent.