Application Note for Quick Freeze/Deep Etch
An ultrastructural imaging technique for biological samples, self-assembled collagen and other biomimetic gels or any hydrated polymeric system.

Introduction

Hydrated polymers are used in many biological and non-biological applications. Example materials include polyvinyl alcohol, collagen, and starch. Hydrated polymers also naturally occur in virtually all organisms. Biomimetic hydrated polymer gels and scaffoldings are increasingly used in the development of artificial replacement tissues such as skin, arterial grafts, cornea and spinal disc replacement. They are also used in gel-based drug delivery systems. Standard imaging modalities (Scanning Electron Microscopy and Transmission Electron Microscopy) do not adequately preserve the delicate structure of these gels during the dehydration and/or embedding process. Even the relatively newer technique of Atomic Force Microscopy cannot discern, in high-resolution, the structure of hydrated polymer gels in solution. Polymer scientists and tissue engineers are left with only limited methodologies to view the nanostructure of their systems.

In a classic paper in 1979, Heuser et al. (Heuser, Reese et al. 1979) were able to capture exocytosis of synaptic vesicles in frog neuromuscular junctions by timing stimulation of neurons with the impact of the tissue on a cold metal block. The frozen stimulated tissue was sectioned under vacuum and the water extracted from the frozen surface by etching or sublimation. Platinum/carbon was evaporated onto the etched, exposed tissue to reveal the structure of the neuromuscular junctions and demonstrated synaptic vesicle exocytosis. The exquisite preservation obtained in this way led to the general development and scientific acceptance of Quick Freeze/Deep Etch (QFDE) as a viable tissue sample preparation methodology that minimally perturbs the material by fixation or processing, and allows high resolution viewing of the undamaged structure by electron microscopy. This method works exceptionally well in the study of many hydrated biological and non-biological systems.

Technique

Quick Freezing - Samples of tissue or hydrated gels may be slam frozen on a high purity copper block cooled to liquid nitrogen temperature (-196° C) or plunge frozen in a secondary cryogen (propane) cooled to liquid nitrogen temperature. Softer samples are plunge-frozen to reduce large-scale deformation or disruption of the sample. It is necessary to quickly freeze the sample to create a "vitrified" (ice crystal-free) layer immediately adjacent to the sample surface. For slam-frozen samples, the vitrified layer extends approximately 10-15 microns from the freezing surface, and for the plunge frozen samples, it extends approximately 3 microns from the freezing surface. Naturally, the best preservation is achieved in the vitrified water layer.

Freeze fracture/deep etching - Following rapid freezing, the sample is stored in liquid nitrogen until it is ready for freeze-fracture and deep-etching. The sample is quickly transferred into the high vacuum (10^{-7} torr) environment of a freeze fracture/freeze etch device (CPG has access to a Cressington CFE-60 Freeze Etch System). After a brief period to stabilize the temperature, the sample is then fractured superficially with a microtome at low temperature (-150 to –196° C). This process exposes a clean face of the sample for etching. After fracture, the sample stage is warmed to approximately -100° C while a cold plate (-196° C) is held just over the sample. This configuration creates a temperature gradient which sublimes water out of the fractured sample surface, revealing the *in situ* structure of the moieties embedded in the water matrix (proteins, lipids, proteoglycans, gel-molecules etc.) which comprise the hydrated system under study. However, to actually visualize these exposed structures requires further processing.

Cambridge
Polymer Group, INC.

Consultation, Testing, and Instrumentation for Polymeric Materials

CPGAN 011
56 Roland St.
Boston, MA 02129
(617) 629-4400
fax: (617) 629-9100
info@campoly.com

Replication - The surface of the sample is replicated in the vacuum chamber immediately following the etching process by evaporation of platinum/carbon (in some cases tantalum/tungsten) at an angle (12°-45°) onto the specimen. This method emphasizes the topography of the surface. The direction of the platinum shadowing can be fixed or rotated around the specimen. The grain size (and ultimate resolution) of the deposited metal depends on the temperature of the sample immediately prior to evaporation. The lower the specimen temperature, the better the resolution. Typical resolutions are 2 nm for platinum/carbon and 0.6 nm for tantalum/tungsten. Following deposition of the metal, carbon is evaporated at 90° to strengthen the replica.

Replica isolation – Following replication, the stable contrast enhancing layer of platinum or tantalum/tungsten is adhered to the surface of the frozen specimen. This layer needs to be lifted off of the sample before it can be viewed in the electron microscope. The coated sample is removed from the freeze-fracture apparatus and is placed in a digestant capable of dissolving the sample without harming the replica (typically bleach or chromic sulfuric acid). The replica is then washed in DI water and picked up on electron microscope grids. These grids may then be viewed in a suitable electron microscope.

Applications

Biological – As the method was developed for biological investigation, it is well suited for that application. Figure 1 demonstrates the difference between standard transmission electron microscopy (A) and QFDE (B). In the QFDE image, the structural features of the matrix are well preserved, including lipid vesicles. Figure 2 is an image of collagen/elastin interaction in extracellular membrane. As can be seen, the quality and resolution of the images is remarkable.

Cambridge
Polymer Group, INC.

Consultation, Testing, and Instrumentation for Polymeric Materials

CPGAN 011

56 Roland St.
Boston, MA 02129
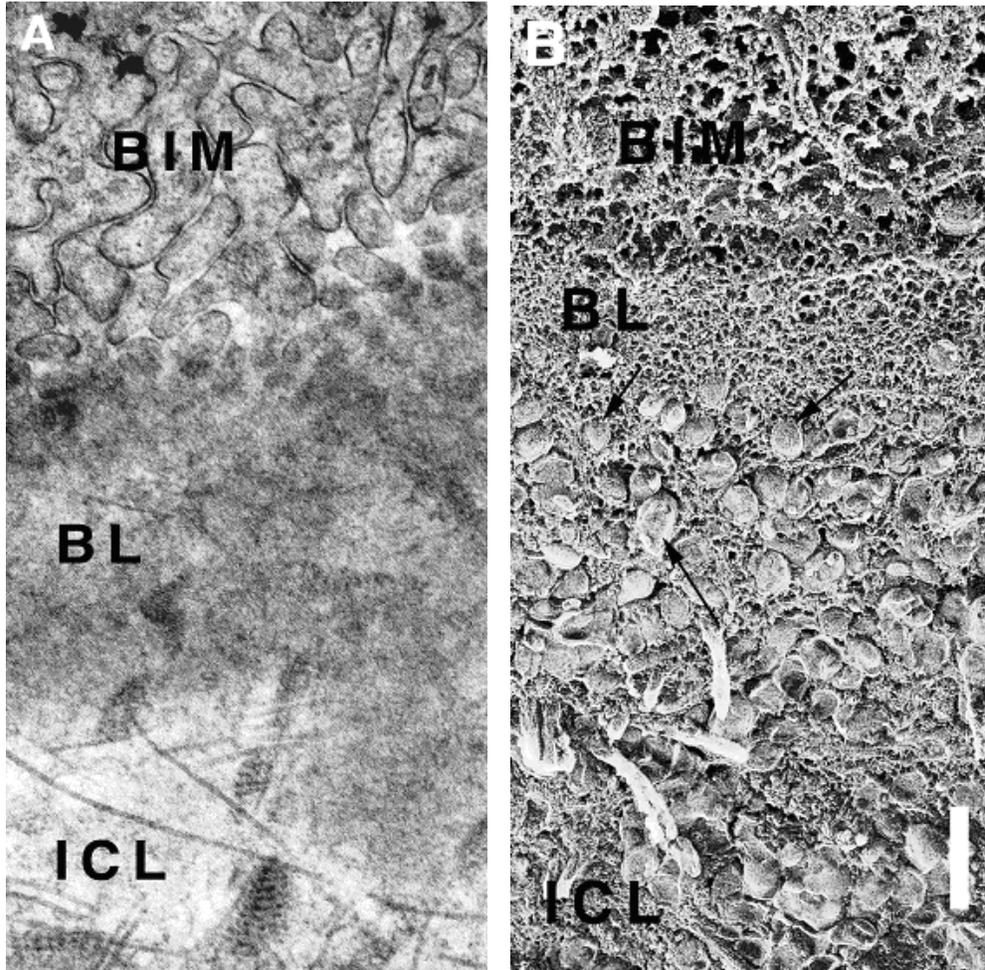(617) 629-4400
fax: (617) 629-9100
info@campoly.com

Figure 1. Standard electron micrograph (A) and QFDE (B) of extracellular matrix. (BL – basal lamina and ICL- collagen layer) adjacent to the membrane of a cell (BIM). Note the large lipid particles embedded in the matrix (arrow; not seen in the standard electron micrograph) and the remarkable detail that is revealed by QFDE. Scale bar is **300 nm.** Photo courtesy Jeff Ruberti
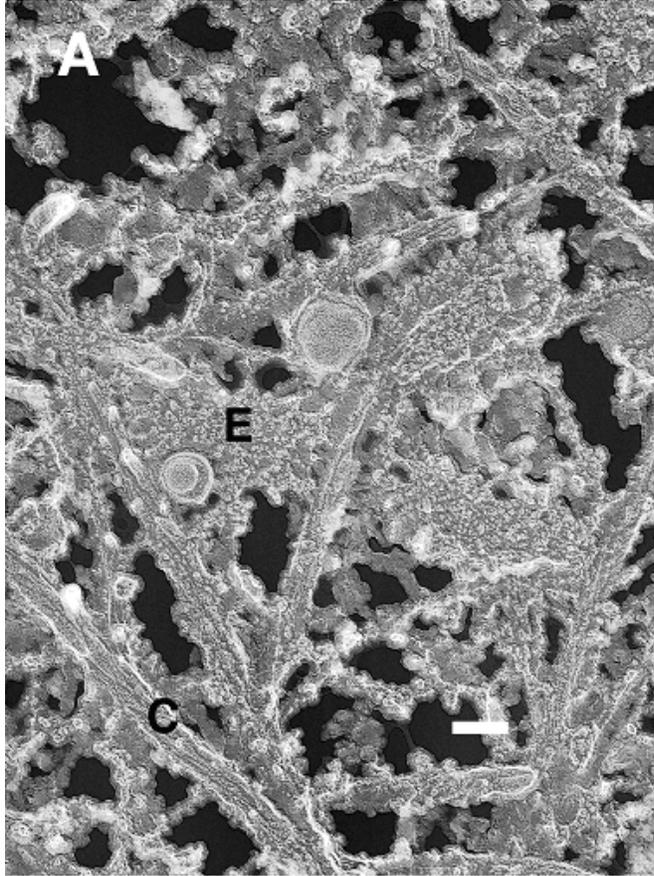
**Cambridge
Polymer Group**, INC.

Consultation, Testing, and Instrumentation for Polymeric Materials

CPGAN 011

56 Roland St.
Boston, MA 02129
(617) 629-4400
fax: (617) 629-9100
info@campoly.com

Figure 2: High magnification QFDE of a collagenous matrix in young human eyes as seen by QFDE. Note the "decoration" of the elastin (**E**) and collagen (**C**) components of the ECM. Scale bar is **100 nm**. At the moment this type of resolution and detail is not achievable by any other method for hydrated gels. Photo courtesy Jeff Ruberti.

Quick Freeze Deep Etch has been used to examine the morphology of a number of biological tissues; however, its use as a tool to determine the engineering properties (permeability) of hydrated matrices has also been proven. Overby et al. 2001 used QFDE to show that the permeability of corneal stroma could be obtained directly from micrographs(Overby, Ruberti et al. 2001).

Non-biological – As mentioned in the introduction, this technique is not limited to use on biological structures alone. Any hydrated polymeric system may be examined using this methodology (including artificial biological scaffolding, hydrogels, drug delivery components etc.). In some cases, QFDE is the only viable approach to imaging the full interaction of structures in a gel-based system. Figure 3 is a picture of a self-assembled peptide hydrogel interacting with a liposome that was thermally triggered to release $CaCl_2$. The structure of the matrix is revealed, as well as the interaction of the liposome with the surrounding gel (even at this low magnification).
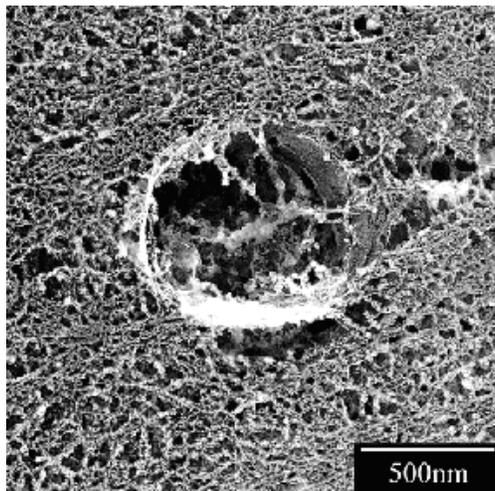
Figure 3. QFDE image of self-assembled gel. Hole was left by liposome, which was thermally modulated to release $CaCl_2$ which triggered self assembly of the surrounding peptide hydrogel matrix *in situ*. Printed with permission of American Chemical Society from (Collier, Hu et al. 2001).

SupraMolecular Agregates- In addition to the imaging of hydrated gels and tissues, supramolecular aggregates may be imaged in solution or on a mica substrate. In Morris et al. 1994 (Morris, Katayama et al. 1994), individual actin filaments were resolved and subjected to 3-D helical reconstruction. They found that the shadowing method accurately preserved the detailed structure of the individual filaments. In Marini et al. 2002 (Marini, Hwang et al. 2002), the structure of a helical beta-sheet, self-assembled peptide was resolved in fine detail (figure 4).
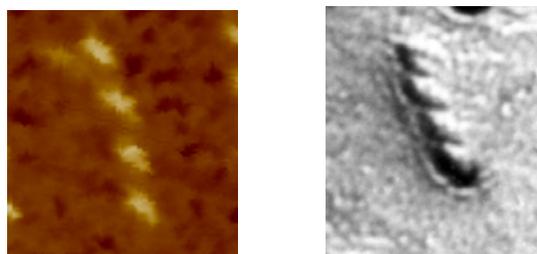

Figure 4. AFM (left) and QFDE micrograph (right)
 of a self-assembled helical beta-sheet of peptide KFE8 (FKFEFKFE)
(Courtesy Davide Marini and Wonmuk Hwang, MIT Microfluidics Lab).

Cambridge Polymer Group, INC.

Consultation, Testing, and Instrumentation for Polymeric Materials

References

Collier, J. H., B. H. Hu, et al. (2001). Thermally and photochemically triggered self-assembly of peptide hydrogels. J Am Chem Soc 123(38): 9463-4.

Heuser, J. E., T. S. Reese, et al. (1979). Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release. J Cell Biol 81(2): 275-300.

Marini, D. M., W. Hwang, et al. (2002). Left-handed helical ribbon intermediates in the self-assembly of a b-sheet peptide. American Chemical Society Nano Letters 415: 14.

Morris, E. P., E. Katayama, et al. (1994). Evaluation of high-resolution shadowing applied to freeze-fractured, deep-etched particles: 3-D helical reconstruction of shadowed actin filaments. J Struct Biol 113(1): 47-55.

Overby, D., J. Ruberti, et al. (2001). Specific hydraulic conductivity of corneal stroma as seen by quick-freeze/deep-etch. J Biomech Eng 123(2): 154-61.