Editorial

An 11-μm-Thick Glycocalyx?
It’s All in the Technique!

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The endothelial glycocalyx is composed of glycoproteins, proteoglycans, and glycosaminoglycans within a high-water-content meshwork overlying the endothelial cells lining blood vessels. This structure maintains the integrity of vascular permeability, protects endothelial cells against leukocytes and red blood cells, and regulates vascular flow and function.1,2 The preservation of the thickness of the glycocalyx is now thought to be an important aspect mediating vascular health; hence, a decrease in thickness may play a role in chronic vascular disease.3 Recently, there has been a discussion in the literature regarding the discrepancy of the glycocalyx measurements in vitro and in vivo.4 Most often, researchers have used traditional transmission electron microscopy (TEM) methods to preserve in vitro glycocalyx, resulting in measurements of less than 100 nm.5 By comparison, recent in vivo imaging studies have demonstrated a much thicker glycocalyx of 8.9 μm.6 To improve on the accuracy of in vitro measurement, researchers are now pursuing additional methodologies to better preserve the hydrated native and gel-like state of the glycocalyx. In this month’s issue of Arteriosclerosis, Thrombosis, and Vascular Biology, Ebong et al7 present a cryofixation technique termed rapid freezing and freeze substitution (RF/FS) and report on the preservation and measurement of an 11-μm-thick glycocalyx.

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The cryotechnique method used by Ebong et al7 is not new, as it was developed back in the 1950s for improved morphological and histochemical preservation of tissues examined by light microscopy.8 Rapid freezing is thought to preserve the native state of biological structures, especially those of high water content, such as the glycocalyx, which contains labile macromolecules. Once cells or tissues are snap-frozen, ice within the specimen is slowly replaced at −90°C by an organic solvent mixed with osmium through a process called freeze substitution. Most importantly, tissues processed by RF/FS have excellent preservation without the use of aldehyde fixation. In the 1970s through the 1990s, progress was made with the addition of cryoprotectants and the development of various ultrarapid freezing methods, reducing structural damage from ice crystals.9 The successful vitrification of the water content present in biological specimens meant the RF/FS technique could be applied to high-magnification TEM studies.10 Initially, specimens thicker than 10 to 15 μm could not be examined, but with the development of a high-pressure freezing apparatus, thicker specimens up to 600 μm could also be processed for TEM. This cryotechnique was primarily used to preserve immunoreactivity in tissue or cells for postembedding immunogold labeling and was used less frequently for morphology-based TEM studies.

The study by Ebong et al7 involved application of the RF/FS TEM technique to rapidly freeze confluent endothelial cell monolayers of macrovascular bovine aortic endothelial cells and microvascular rat fat pad endothelial cells at −90°C with freeze substitution in acetone containing 1.0% osmium tetroxide for added contrast. Once this was completed, the specimens were slowly raised to room temperature, embedded into LX112 epoxy resin, and polymerized. The embedded cells were thin sectioned perpendicular to their growth plane, stained with uranyl acetate and lead citrate, and then imaged and analyzed by TEM. The authors reported glycocalyx thicknesses of 11 and 6 μm from bovine aortic endothelial cells and microvascular rat fat pad endothelial cells, respectively.

They compared their imaging results of glycocalyx thickness to specimens prepared by traditional TEM methods. The cell monolayers were fixed in 2.5% glutaraldehyde containing ruthenium red (developed to preserve the glycocalyx for TEM in 196611), postfixed in 1.0% osmium tetroxide, dehydrated in alcohol, embedded in LX112, and thin sectioned for TEM analysis. Ebong et al’s traditional TEM processing results produced an expected 42-nm-thick glycocalyx measurement, which stands in strong contrast to their cryotechnique findings of an 11-μm-thick glycocalyx.7 They postulated that it was either the fixation with a cross-linking aldehyde or the alcohol dehydration process that “collapsed” the glycocalyx, resulting in the thin, electron-dense layer typically observed in traditional TEM studies.

Surprisingly, before the work of Ebong et al,7 no researcher had thought of applying this technique for glycocalyx preservation, despite decades of glycocalyx imaging by TEM. Their results are timely, given the recent debate in the literature negating the usefulness of human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cell cultures tested during in vitro flow-mediated mechanotransduction experiments. Other researchers, using cylindrical collagen microchannels and fluorescent microparticle image velocimetry, had demonstrated a greatly diminished bovine aortic endothelial cells and human umbilical vein endothelial cell glycocalyx of 0.02 and 0.03 μm thickness, respectively.
These authors concluded the glycocalyx to be “absent in vitro” when compared with their in vivo 0.52-μm measurement. A year later, another group of researchers attempted to address this controversy. They examined ex vivo fresh human umbilical cords and primary human umbilical vein endothelial cell cultures by TEM. Both specimen types were processed using traditional TEM methods; however, instead of using ruthenium red, they mixed lanthanum nitrate with their glutaraldehyde fixative. The perfusion-fixed umbilical vein glycocalyx measured an average 878 nm, in contrast to an in vitro cultured human umbilical vein endothelial cell average thickness of 29 nm. The disparity of these researchers’ results compared with Ebong et al’s cryotechnique measurements provides yet another dimension to this ongoing controversy.

A myriad of experiments await investigators wishing to exploit the RF/FS TEM technique for the study of glycocalyx biogenesis and molecular organization both in vitro and in vivo. Regarding the former, Ebong et al have begun by examining the enzymatic digestion of the glycocalyx with heparinase in cultured cells. After digestion, the 11-μm thickness was maintained, but the morphology was altered to a scaffold-like open matrix containing sparse vertical fibrils. Adapting the RF/FS TEM technique for postembedding immunogold labeling may build on Ebong et al’s work by enhancing our understanding of the structural organization of normal or damaged glycocalyx. Similarly, another approach to investigating changes in glycocalyx structure could involve applying the RF/FS TEM technique to in vitro flow conditions. This perspective may reveal the hidden mechanics of glycocalyx flattening that are not observable under static culture conditions.

The RF/FS TEM technique may also be useful to study the glycocalyx in vivo. It now appears that traditional TEM studies of the glycocalyx have always documented it in a collapsed state. Examination of specimens prepared via RF/FS TEM may provide additional morphological details needed to advance our understanding of the glycocalyx and its involvement in vascular flow studies. For instance, chemokine-stimulated leukocyte behavior within a better preserved glycocalyx observed under the TEM may offer more insight into leukocyte movement than found in previous transmigration studies. The mouse cremaster muscle is commonly used in transmigration experiments and would be a good candidate for RF/FS TEM because it is usually pinned out and very thin (400 to 500 μm thick; personal observation). However, one would need to use a high-pressure freezing apparatus to uniformly freeze such a thick specimen. Thus, the remarkable results of Ebong et al bring to the forefront the utility of the RF/FS TEM technique some 53 years after its inception. The use of TEM cryotechniques to advance higher quality morphological studies of cells or tissue in their native state may be more relevant than ever. This is true not just in vascular-related research but in other fields, such as embryology or neuroscience, as specimens from these types of tissue also have intrinsically high water content. The ability to preserve and examine the hydrated states of tissue structure at high magnifications may broaden our understanding of normal and pathophysiologic processes.

References

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