Glycerinated Glycol Methacrylate Mounting Medium in Highlighting Non-Coated or Glycerin-Substituted Microsamples under the Scanning Electron Microscope

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Summary. Glycol methacrylate hardened with 10-15% glycerin showed no charging and gave dark contrast in the scanning electron microscope with an acceleration voltage of 25 kV and a specimen current of $1 \times 10^{-10}$ A. Using human blood cells, it was shown that this conductively treated resin is a useful mounting substrate to highlight the non-coated microsamples or to obtain their highly contrasted images under the scanning electron microscope. It was also demonstrated that the glycerin-substituted blood cells can be scanned with little shrinkage.

Our previous paper demonstrated that glycol methacrylate was useful for embedding tannin-osmium conductive samples for scanning observation (Murakami, 1975). Our recent experiments have revealed that resin, when hardened with glycerin, becomes conductive and represents an excellent mounting medium. It highlights non-coated microsamples, including the glycerin-substituted blood cells, under the scanning electron microscope. This paper will report the technique and result of this new method.

Materials and Methods

Glycol methacrylate (2-hydroxyethyl methacrylate, GMA) monomer was mixed with 10-15% glycerin. After supplementing with 0.3-0.5% benzoyl peroxide (catalyst for polymerization), the glycerinated glycol methacrylate was hardened in an incubator (45°C for 1 day and 60°C for 2-3 days). The hardened resin was cracked with chisels and the blocks with sharply and homogeneously flattened surfaces were prepared.

The human blood cells were obtained by centrifugation of the venous blood of a healthy 37-year-old man and fixed for 6 hrs with 1.0% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) (Tokunaga et al., 1969). They were treated as follows; (1) dehydrated as usual through a graded series of alcohol and isoamyl acetate and dried in a critical point drier (HCP-1) using carbon dioxide (Hattori, 1972; Al-Samarrai, 1975), (2) intensely osmicated by the revised tannin-osmium method (Murakami, 1974) and dried in the critical point drier after dehydration with alcohol and isoamyl acetate, or (3) immersed in a graded series of glycerin and suspended, without drying, in glycerin (cf. Tanaka, 1969).

Non-coated human blood cells thus critical point-dried or suspended in glycerin were mounted on those sharply flattened surfaces of the glycerinated glycol methacrylate resin blocks and observed, without any metal coating, under a scanning electron microscope (JSM-U3) using an acceleration voltage of 25 kV and a specimen
current of $1 \times 10^{-10}$ A.

In order to check the results of scanning, some specimens were dispersed before fixation in the physiological saline solution and their diameters were calculated by light microscopy.

Fig. 1. A survey scanning view of the glycerin-substituted human blood cells mounted on the glycerinated glycol methacrylate resin block with conductivity. Glycerin is readily sublimated in the vacuum column of the scanning electron microscope. By this removal of superfluous glycerin, the blood cells are exposed in which glycerin still fully remained. G glycerin area where the suspended blood cells can be seen through glycerin with gray contrast, S sublimated area where the blood cells were completely exposed without superfluous glycerin. Note that the exposed blood cells are markedly highlighted in the dark background of the conductive resin block though they are neither stained nor coated with any metal. Acceleration voltage 25 kV, tilting angle 0°. $\times 170$. 
Results and Discussion

The diameters of the non-fixed red blood cells observed by light microscopy measured 7.3-8.2 µ.

The glycerinated glycol methacrylate mounting medium described above showed no charging in the scanning electron microscope with the 25 kV acceleration voltage and the 1 × 10⁻¹⁰ A specimen current. The secondary electron emission of this conductive resin block was poor and gave dark contrast under the scanning electron microscope (Fig. 1).

Fig. 2. A closer scanning view of the non-conductive blood cells mounted on the glycerinated glycol methacrylate resin block. The blood cells are penetrated by the scanning electron beam using 25 kV acceleration voltage and can be viewed, as in cases treated by glycerin substitution and osmium impregnation (Fig. 1, 3, 4), without charging. Note that the blood cells are beautifully highlighted in the dark background. Note also that the surfaces of the blood cells are sufficiently contrasted. Acceleration voltage 25 kV, tilting angle 0. ×9,000
Though the dried specimens were immediately observed, in the glycerin-suspended cases preliminary sublimation in the vacuum column of the scanning electron microscope was necessary to remove the superfluous glycerin or to expose the suspended blood cells (Fig. 1). The conductive blood cells treated by the osmication or glycerin-substitution showed no charging (Fig. 1, 3, 4). The non-conductive blood cells with neither osmication nor glycerin-substitution were readily penetrated by the scanning electron beam using the 25 kV acceleration voltage (see below) and could also be viewed without charging (Fig. 2).

In all cases including the non-osmicated ones with weak secondary electron emission, the blood cells were beautifully highlighted in the dark background of the resin and sufficiently contrasted for observation of their surface details (Fig. 2–4). Even in the glycerin-substituted cases where the images were rather obscured by the contamination with sublimated glycerin, the fine microvilli of white cells were clearly visible (Fig. 4).

Fig. 3. A closer scanning view of the tannin-osmium impregnated blood cells mounted on the glycerinated glycol methacrylate resin block. Note that when secondary electron emission is enhanced by intense osmication, the images are sharpened. Acceleration voltage 25 kV, tilting angle 0. ×9,000
Marked differences in cell size were noticed, reflecting the different methods of preparation. The red blood cells with neither osmication nor glycerin-substitution measured 5.4–6.6 \( \mu \) in diameter (Fig. 2), and the tannin-osmium impregnated ones 5.6–7.1 \( \mu \). In contrast to these dried samples, the glycerin-substituted red blood cells were calculated to be 7.2–8.1 \( \mu \), which nearly corresponded to the value estimated by the light microscopy of the living cells in the physiological saline solution (see above). The similar differences in size were also noticed in the white cells as well as in their microvilli. As seen in Figures 3–5, the villi of the dried neutrophils were emaciated with thorn-like appearance, while those of the glycerin-substituted ones were rather stout but exquisite.

This paper shows that the glycol methacrylate, when hardened with glycerin, is assured of conductivity and excellently used as a mounting medium to highlight the non-coated microsamples or to give their beautifully contrasted images with dark background under the scanning electron microscope. The similar highlighting or contrasting of the non-coated microsamples have also been attempted by other authors. They used light metals such as carbon and aluminum in order to observe Latex particles (Broers and Brandis, 1969), dust samples (DeNee, 1971) and Escherichia coli affected with T4-phage (Amako, K., 1973, cited by Watanabe et al., 1975). Horisberger

**Fig. 4.** Closer view of the glycerin-substituted blood cells mounted on the conductive resin block. Although the images are rather obscured by the sublimated glycerin, the microvilli of the cells are sufficiently observed. Note that the cells show little shrinkage and that the microvilli are stout in contrast to the dried and emaciated ones shown in Figure 3. Acceleration voltage 25 kV, tilting angle 0. \( \times 9,000 \)
et al. (1975), who brightened the gold particles immunologically reacted with *Candida utilis* cell and dramatically demonstrated the advantage of the non-coating observation, used copper adhesive as the background substrate. Our preliminary experiments in which carbon was evaporated on the present resin block and observed in the scanning electron microscope, however, suggested that our resin is less emissive of secondary electron than those metals and is more useful to contrast the non-coated biological specimens with weak secondary electron emission. The cellophane sheet, which seemed as little emissive as our resin and was used by DeNee (1971) in mounting dust samples, tended to charge at the 25kV acceleration voltage though it contained 10–15% glycerin.

In the use of the glycol methacrylate, concentration of glycerin should be adjusted to 10–15% because our preliminary experiments showed that the glycol methacrylate hardened with 5.0% glycerin was charged at the 25kV acceleration voltage and that the resin containing 20% glycerin was easily sublimated or damaged by the scanning electron beam at this voltage. It is further noted that when an acceleration voltage of 5kV was used, the blood cells with no conductive treatment tended to charge. This will confirm that the charging of these non-conductive specimens depends on the acceleration voltage or the penetrating power of the scanning electron beam. In such charged cases the charge-balance method, in which the specimens are observed with tilting angles of 45 or more degrees (Ichinokawa, T., 1974, cited by Watanabe et al., 1975; Horisberger et al., 1975), was recommended.

This paper also proves that the glycerin-substitution, which was introduced by Tanaka (1969) in the scanning of the blocked lens fibers of the fish eye and lately applied by Panessa and Gennaro (1972) in the observation of fragile botanical tissues, is of great value even in the observation of the non-coated microsamples. As described above, the glycerin-substituted red blood cells had similar diameters to those in the physiological saline solution. This confirms that the glycerin-substituted neutrophil with graceful villi shown in Figure 4 represents a more natural form than the dried ones with thorn-like processes in Figure 3.

Our previous paper showed that glycol methacrylate is a suitable embedding substrate of the tannin-osmium conductive samples for scanning demonstration of delicate tissue elements such as the transmural blood cells (Murakami, 1975). Needless to say the glycerin method herein described is also excellent for such embedding and even grounds the isolatedly embedded specimens to avoid their charging.

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走査電子顕微鏡下に無蒸着あるいはグリセリンを植え込んだ微小試料を輝かすための加グリセリングリコールメタクリレート載台

村上宅郎 と 入野昭三

10〜15％にグリセリンを加えて硬化したグルコールメタクリレートは、加速電圧25kV,
Glycerinated GMA Mounting Medium for Non-Coated SEM Specimens


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