New Embedding Method Employing GMA and Quetol 523 for Light and Electron Microscopic Observations of Semi-thin Sections

By

TSUYUKA KUSHIDA, YASUKAZU NAGATO
and HIROSHI KUSHIDA*

Department of Anatomy, School of Medicine, Tokai University, Isehara 259-11,
Japan, *Electron Microscope Laboratory, Keio University,
Shinjuku-ku, Tokyo 160, Japan

—Received for Publication, September 28, 1978—

Key Words: Glycol methacrylate-Quetol 523, Polymerization, Semi-thin section, Osmium tetroxide vapor staining.

Summary. A new method, employing water-miscible methacrylates, for light and electron microscopic observations of semi-thin sections was devised for ease in embedding, infiltration, sectioning and staining. The method used purified GMA (glycol methacrylate) and Quetol 523 (methoxy polyethylene glycol 400 methacrylate), and QCU-1 (2,2'-azobis isobutyronitrile paste) as catalyst. A mixture of these could be polymerized at 60°C for about 12 hr or at 39°C for about 36 hr to produce a block with excellent cutting properties. Blocks of these resins were easy to section.

Tissues were fixed in 2.5% glutaraldehyde with buffered phosphate at pH 7.4 for 3 hr. Sections 0.3-2 μ m thick were cut with glass knives on a conventional ultramicrotome. They could be attached to slide glasses or grids by water flotation, without adhesive. They should be dried at room temperature. Staining with aqueous solutions of basic and acid dyes, without removing the embedding matrix, was sharp and brilliant as usual. These stained sections were observed with a light microscope. The same parts of such sections, 0.3-0.5 μ m thick, could be examined with an accelerating voltage of 100 kV at low magnification (300-1,500 times) using a conventional electron microscope. Thus, photomicrographs and electron micrographs of the same part of a tissue sample could be exactly compared.

The resolution of the electron microscope was so high that cytoplasmic components were easily identified in the cytoplasm. Osmium tetroxide vapor staining gave better contrast in the images of the specimens.

Introduction

Recently, several glycol methacrylate (GMA = 2-hydroxyethyl methacrylate, HEMA) embedding methods^{1, 2, 3, 8, 11, 12, 13)} have been used for studying the fine structure of cells and tissues by light

microscopy in place of the conventional paraffin embedding method. The embedding mixtures in these methods commonly used GMA, plasticizer (polyethylene glycol 400, 2-n-butoxyethanol, etc.) and catalyst. The final hardness of the polymerized blocks was controlled with

a volume of plasticizer. Thus, the cutting properties of the polymerized blocks were reduced.

Our method employed purified GMA and Quetol 523 (methoxy polyethylene glycol 400 methacrylate) as water-miscible methacrylates, and QCU-1 (2,2'-azobis isobutyronitrile paste) as catalyst, a mixture of which could be hardened at excellent cutting properties. The hardness of the polymerized resins could easily be adjusted by altering the ratio of the components to suit the specimens embedded in the mixture. Sections 0.3–2 μm thick could easily be cut with glass knives on a conventional ultramicrotome. Staining with aqueous solutions of basic and acid dyes, without removing the embedding matrix, was sharp and brilliant as usual. These stained sections were observed with a light microscope. The same parts of such sections, 0.3–0.5 μm thick, could be examined with an accelerating voltage of 100 kV at low magnification (300–1,500 times) using a conventional electron microscope. The contrast in images of the specimens could be increased by exposure to osmium tetroxide vapor.

Materials and Methods

1. Specimens

Fresh tissues (liver, pancreas, small intestine, kidney, salivary gland and testis) excised from adult male I.C.R. mice were used. The tissue sample sizes were ca. 7×5×3 mm for light microscopy and ca. 3×3×1 mm for electron microscopy.

2. Fixation

The pieces were fixed in 10% phosphate-buffered formalin (pH 7.2), 2.5% phosphate-buffered glutaraldehyde (pH 7.2), Zenker's fixative, Bouin's fixative, Carnoy's fixative, etc. for 6 to 24 hr at 0°–4°C, and washed in the buffer for 3

to 6 hr for light microscopy. For electron microscopy, the pieces were fixed in 2.5% phosphate-buffered glutaraldehyde (pH 7.2) for 2 to 3 hr at 0°–4°C, and washed in the buffer for 1 hr.

3. Dehydration

After washing, they were dehydrated in the ordinary way in 50%, 70%, 80%, 90%, 95%, and two changes of 100% alcohol for 30 min each.

4. Embedding

As mentioned, the embedding method used the water-miscible methacrylates, purified GMA and Quetol 523*, and QCU-1 as catalyst.

The following mixtures (A, B, C and D) were suitable for general embedding:

	A	B	C	D
	(for light microscopy)		(for electron microscopy)	
GMA	80 ml	80 ml	85 ml	85 ml
Quetol 523	20 ml	20 ml	15 ml	15 ml
QCU-1	0.05 g	0.4 g	0.05 g	0.4 g

These mixtures set in about 12 hr at 60°C (A and C) and in about 36 hr at 39°C (B and D) and yielded clear, colorless blocks. The mixtures A and B were suitable for cutting 1–2 μm sections. In the case of embedding with C or D, it was easy to cut 0.3–0.5 μm sections.

After dehydration, the following infiltration procedure was appropriate. All steps were carried out on a shaker⁴⁾ at room temperature.

- 1) 100% alcohol/GMA-Quetol 523 mixture (1:1) 1–2 hr
- 2) GMA-Quetol 523 mixture 2–4 hr
- 3) GMA-Quetol 523 mixture 2–4 hr
- 4) Embed in gelatin capsules

Gelatin capsules were filled to the brim with GMA-Quetol 523 mixture,

* Sold by Nissin EM Co., Ltd., Yotsuya Sangyo Bldg., 2, Samon-cho, Shinjuku-ku Tokyo 160, Japan; and Tedpella, Inc., P.O. Box 510, Tustin, Calif. 92680, U.S.A.

and the specimens were introduced. Polymerization was achieved in about 12 hr at 60°C (A and C) or in about 36 hr at 39°C (B and D).

5. Trimming and Sectioning

After removal of the gelatin capsule, the block was placed in a holder and observed through a dissecting microscope to aid precise trimming. The block face was carefully trimmed to a maximum size of ca. 6×4 mm or ca. 2×2 mm with a clean, sharp razor blade. The trimmed block was cut with a dry glass knife having a thickness of an approximately 10 mm thick on an ultramicrotome (Porter-Blum MT-1 or MT-2B) or JB-4 Porter-Blum microtome. Sections were generally cut 1–2 μm in thickness for light microscopy and 0.3–0.5 μm in thickness for electron microscopy.

After cutting at slow speed, each section was lifted from the knife edge with a pair fine-pointed watch-maker's forceps and placed on a clean distilled water surface at room temperature. It usually spread flat on the surface. It was then moved to the middle part of the water surface by blowing with a gentle blast of clean dry gas from a freon gas spray, and was ready for mounting on a microscope slide after a few minutes. For electron microscopy, a 50–100 mesh rhodium coated grid was pressed gently over the floating sections, as shown in Figure 1. The excess water on the microscope slide or grid was blown off with a quick blast of clean dry gas from a freon gas spray. The sections were then allowed to dry at room temperature. They adhered to the slides or the grids without adhesive during staining and washing.

6. Staining

The sections were stained with aqueous solutions of basic and acid dyes for light microscopy without removing the embedding matrix. The staining time

Fig. 1. Method of mounting a section on a grid. a: Section, b: A gentle blast of freon gas, c: Gas spray, d: Forceps, e: Grid, f: Vial, g: Distilled water.

required was longer than that for paraffin sections.

A drop of staining solution was placed on the section for a given length of time. The excess stain solution was then rinsed away with a jet of tap water, and a drop of the next staining solution was added in the same way. After a final rinsing in distilled water, the excess water on the slide was blown off with a quick blast of clean dry gas from a freon gas spray. The sections were covered with a cover glass and mounted with Diatex or Eukitt.

For staining sections on grids, the following method was used to facilitate handling of the grids⁹. A short piece of polyethylene tubing was employed as a grid-holder. The most suitable tubing was a white type of slightly hard quality, about 2.7 mm in outer diameter, about 1.7 mm in inner diameter and with a wall thickness of about 0.5 mm. One end of the tubing was slit to a depth of about 3 mm with a thin razor blade, as shown in Figure 2A. The tubing was washed in alcohol with an ultrasonic generator and then dried at room temperature.

In order to insert the grids into the holder, the slits were opened slightly

Fig. 2. Method for inserting grids in slits in the polyethylene tubing. a: Polyethylene tubing, b: Half of double edge razor blade, c: Glass rod, d: Slit, e: Forceps, f: Grid.

by inserting a sharp glass rod into the end of the tubing (Figure 2B). A grid was fixed into the opened slit on one side to a distance of about 0.3 mm from the edge of the grid (Figure 2C, D).

Application of a slight torque when the second grid was inserted into the slit on the other side, served to hold the first grid firmly in place (Figure 2E, F, G).

For staining, the grids were firmly inserted into the small slits of the polyethylene tubing, as described above, and pieces of the polyethylene tubing were attached to sharp glass rods of a cap, as shown in Figure 3A. The sections were stained with aqueous solutions of basic and acid dyes without removing the embedding matrix.

The grids with sections were placed

Fig. 3. Staining using the polyethylene tubing as grid-holder. a: Cap, b: Glass rod, c: Polyethylene tubing, d: Slit, e: Grid with section affixed, f: Staining solution, g: Osmium tetroxide.

into a staining solution for a given length of time. After staining, they were washed with distilled water and treated with another solution in the case of double staining without drying. After washing with distilled water, the pieces of polyethylene tubings were removed from the glass rods, rinsed carefully with a stream of distilled water directed along the axis of the tubing, and then dried with a gentle jet of freon gas applied in the same direction.

For osmium tetroxide vapor staining⁷⁾, the pieces of polyethylene tubing with the grids were attached to sharp glass rods of a cap, as shown in Figure 3B. Next, the crystal of osmium tetroxide was placed at the bottom of a vial. The cap with the pieces of polyethylene tubing was placed on the vial and the sections on the grids were exposed to osmium tetroxide vapor for 6-12 hr.

7. Examinations

The stained sections were observed with a light microscope. The same parts of such sections, 0.3-0.5 μm thick, were examined with an accelerating voltage of 100 kV at low magnification using JEM-100C and HU-12AS Electron Microscopes. Electron micrographs were prepared at low magnification (300-1,500 times) and enlarged photographically to a magnification of ca. 3,000 times.

Results and Discussion

Several glycol methacrylate embedding methods^{1, 2, 3, 8, 11, 12, 13)} for light microscopy have used GMA, polyethylene glycol 400 or 2-n-butoxyethanol as plasticizer and 2,2'-azobis isobutyronitrile or benzoyl peroxide as catalyst. The final hardness of the polymerized blocks was controlled with a volume of plasticizer. Thus, the cutting properties of the polymerized blocks were reduced. After placing the section on the slide, and heating on a

hot plate at 60°-100°C, the section adhered to the slide.

Our embedding method employed purified GMA and Quetol 523 as water-miscible methacrylates, and QCU-1 as catalyst. Quetol 523 was a light-colored, flexible, water-miscible methacrylate. It had a low viscosity, and reacted with GMA to become an integral part of the polymerized system. QCU-1 was readily miscible with the methacrylates.

The GMA-Quetol 523 mixtures (A, B, C and D) set in about 12 hr at 60°C and in about 36 hr at 39°C. They had a low viscosity, and were therefore easy to handle and penetrated readily and completely into the specimen, producing a homogeneous block from which it was easy to cut 0.3-2 μm sections. The final hardness of the block could easily be adjusted by altering the ratio of GMA and Quetol 523 to suit the specimen. Mixtures of GMA and Quetol 523 in ratios of 80:10 and 85:15 were suitable for sectioning at 20°C.

A gelatin capsule with a flat bottom was appropriate for flat embedding of a specimen. The bottom of the gelatin capsule was heated on a hot plate at ca. 90°C and pressed with a flat section of metal rod from the inside of the gelatin capsule. This was then cooled at room temperature. A polyethylene capsule or cap was not used for embedding. The polymerized block remained soft on the sides and bottom, where it was in contact with the polyethylene capsule or cap, even though it might be hard in the middle. Moreover, the resin mixture on the exposed upper surface did not polymerize properly under these conditions, since polymerization of mixture did not proceed to completion in the presence of air.

Tissues were fixed in formaldehyde and glutaraldehyde fixatives, Zenker's fixative, Bouin's fixative, Carnoy's fixa-

tive, etc. However, fixatives containing OsO_4 could not be used, since polymerization was retarded or even inhibited in the presence of tissues preserved in such fixatives.

Sections $0.3\text{--}2\text{ }\mu\text{m}$ thick could easily be cut with glass knives on a conventional ultramicrotome. The sections on the slide or the grid were allowed to dry at room temperature. They adhered to the slide or the grid without adhesive during staining and washing.

For staining sections on grids, a method employing a short piece of polyethylene tubing as a grid-holder was suitable for ease in handling the grids. Staining with aqueous solutions of basic and acid dyes, without removing the embedding matrix, was sharp and brilliant as usual, as shown in Figure 4—Figures 12A and 13A. Figure 12A depicts mouse small intestine, stained with hematoxylin and eosin, while Figure 13A depicts mouse seminiferous tubules treated with osmium tetroxide vapor after toluidine blue staining. Figures 12B and 13B show electron micrographs of the same parts of the sections as in Figures 12A and 13A, respectively. Figures 12C and 13C are enlargements from Figures 12B and 13B, respectively. Photomicrographs and electron micrographs of the same parts of the tissues could thus be compared exactly.

Figure 14 shows an unstained section of mouse seminiferous epithelium in the testis. Figure 15 depicts mouse salivary gland, stained with osmium tetroxide vapor. Figure 16 depicts mouse proximal convoluted tubule of the kidney, stained by the Giemsa method. Figure 17 shows mouse seminiferous epithelium in the testis, stained with hematoxylin and eosin and post-stained with osmium tetroxide vapor. Figure 18 shows mouse small intestine, treated with osmium tetroxide vapor after hematoxylin and

eosin staining. Figure 19 is an enlargement from Figure 18.

In Figures 12, 14, 16 and 17, the various images for no staining, the Giemsa method and hematoxylin and eosin staining show good contrast. In Figure 12B, specific tissue contrast was based on the selected color of the dye at the light microscopic level. In Figure 13B, specific contrast at the intercellular boundaries distinguished each cell and the cytoplasmic components were clearly recognizable. The resolution of the electron microscope was so high that cytoplasmic components, particularly mitochondria, Golgi apparatus and endoplasmic reticulum were easily identified. The cellular structure of the striated border of intestinal epithelial cells, the brush border or proximal tubules of the kidney and desmosomes of the seminiferous epithelium in the testis were also clearly demonstrated under the electron microscope. Images from osmium tetroxide vapor staining showed a distinctly better contrast.

Acknowledgement

The authors wish to thank Mr. H. Iwata and Mr. S. Aita, JEOL Ltd., Aki-shima, for their kind assistance in this work.

References

- 1) Ashley, C.A. and Feder, N.: Glycol methacrylate in histopathology. *Arch. Pathol.*, **85** 391-397, 1966.
- 2) Bennett, H.S., Wyrick, A.D., Lee, S.W. and McNeil, J.H.Jr.: Science and art in preparing tissues embedded in plastic for light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. *Stain Technol.* **51**: 71-97, 1976.
- 3) Cole, M.B.Jr. and Sykes, S.M.: Glycol methacrylate in light microscopy: a

- routine method for embedding and sectioning animal tissues. *Stain Technol.* 49: 387-400, 1974.
- 4) Kushida, H.: A new rotary shaker for fixation, dehydration and embedding. *J. Electron Microscopy.* 18: 137-139, 1969.
 - 5) Kushida, H.: New device for staining thin sections. *J. Electron Microscopy.* 21: 201-202, 1972.
 - 6) Kushida, H.: A new method for embedding with GMA and Quetol 523 for electron microscopic observations on semi-thin sections for light microscopy. *J. Electron Microscopy.* 26: 351-353, 1977.
 - 7) Kushida, H.: Handling of semi-thin sections of GMA-Quetol 523 embedded tissue for light and electron microscopy with special reference to osmium tetroxide vapor staining. *J. Electron Microscopy.* 27: 59-60, 1978.
 - 8) Kushida, T. and Kushida, H.: Preparation of 1-2 micron sections of plastic embedded tissues for light microscopy. *J. Tokyo Woman's Medical College.* 45: 227-230, 1975.
 - 9) Kushida, T., Nagato, Y. and Kushida, H.: A new embedding method employing water-miscible methacrylates for light microscopy. *Acta Anatomica Nipponica.* 52 1-9, 1977.
 - 10) Kushida, T., Nagato, Y. and Kushida, H.: Observation on the same place in semi-thin section with both light and electron microscopy. *J. Electron Microscopy.* 26: 345-348, 1977.
 - 11) Ruddell, C.L.: Hydroxyethyl methacrylate combined with polyethylene glycol 400 and water; an embedding medium for routine 1-2 micron sectioning. *Stain Technol.* 42: 119-123, 1967.
 - 12) Ruddell, C.L.: Embedding media for 1-2 micron sectioning. 2. Hydroxyethyl methacrylate combined with 2-butoxyethanol. *Stain Technol.* 42: 253-255, 1967.
 - 13) Ruddell, C.L.: Embedding media for 1-2 micron sectioning. 3. Hydroxyethyl methacrylate-benzoyl peroxide activated with pyridine. *Stain Technol.* 46: 77-83, 1971.

Explanation of Figures

Plate I

- Fig. 4. Mouse testis fixed in Bouin's fixative and embedded in GMA-Quetol 523 mixture. One micron section is stained with hematoxylin and eosin.
- Fig. 5. Mouse muscle fixed in Bouin's fixative and embedded in GMA-Quetol 523 mixture. One micron section is stained with hematoxylin and eosin.
- Fig. 6. Mouse kidney fixed in Bouin's fixative and embedded in GMA-Quetol 523 mixture. One micron section is stained with hematoxylin and eosin.
- Fig. 7. Mouse kidney fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. One micron section is stained with hematoxylin and eosin.

Plate II

- Fig. 8. Mouse submandibular gland fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. Two micron section is stained with Mallory-Azan.
- Fig. 9. Mouse kidney fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. One micron section is stained with acid fuchsin.
- Fig. 10. Mouse kidney fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. One micron section is stained with toluidine blue.
- Fig. 11. Mouse liver fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. Two micron section is stained with methylene blue.

Plate III

Fig. 12A. Mouse small intestine fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is stained with hematoxylin and eosin. $\times 900$

Fig. 13A. Mouse seminiferous tubules fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is treated with osmium tetroxide vapor after toluidine blue staining. $\times 900$

Figs. 12B and 13B. Electron micrographs of the same places in the sections as Figs. 12A and 13A, respectively. $\times 900$

Plate IV

Figs. 12C and 13C.

Electron micrographs, enlarged from Figs. 12B and 13B, respectively. $\times 1,500$

Plate V

Fig. 14. Mouse seminiferous tubules fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is unstained. $\times 3,000$

Fig. 15. Mouse salivary gland fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is stained with osmium tetroxide vapor. $\times 2,300$

Plate VI

- Fig. 16. Mouse proximal convoluted tubules in the kidney fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is stained by the Giemsa method. $\times 3,000$
- Fig. 17. Mouse seminiferous epithelium in the testis fixed in 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is stained with hematoxylin and eosin and post-stained with osmium tetroxide vapor. $\times 3,000$

Plate VII

Fig. 18. Mouse small intestine fixed 2.5% glutaraldehyde and embedded in GMA-Quetol 523 mixture. 0.3 micron section is treated with osmium tetroxide vapor after hematoxylin and eosin staining. $\times 3,000$

Fig. 19. Electron micrograph enlarged from Fig. 18. $\times 6,000$