

CROSS-LINKED POLYAMPHOLYTES

New Water-Soluble Embedding Media for Electron Microscopy

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In connection with an investigation of the staining of intracellular antigens with ferritin-antibody conjugates (1), we required an embedding material which was extensively ionic. Although several hydrophilic embedding materials have been described (*cf.* reference 2), to our knowledge no satisfactory ionic ones have been reported. We sought to prepare an ionic embedding material by the bulk polymerization of a suitable monomer or monomers. The simplest kind of ionic polymer is a *polyelectrolyte* (3), which may be prepared by polymerizing an ionizable monomer such as methacrylic acid (MA). A polyelectrolyte thus contains ionic charges of only one sign. It occurred to us that it would be more advantageous, for a variety of reasons, to prepare a *polyampholyte* (4), made by the copolymerization of comparable molar quantities of two monomers of opposite ionic charge. A polyampholyte is studded with more or less alternating negative and positive charges which confer on it a large *total* ionic charge but only a small *net* ionic charge. In this respect, a polyampholyte resembles a protein molecule, or the surface of a biological membrane.

Fortunately, the copolymerization of a number of such pairs of vinyl monomers has already been carefully investigated (4-6). Such copolymers are usually water-soluble, so that for tissue embedding it would ordinarily be necessary to add a cross-linking agent to the monomers to render the copolymer insoluble. We have made cross-linked vinyl-type polyampholytes from the anionic monomer MA and the cationic monomer dimethylaminoethylmethacrylate (DMA), together with a small amount of a cross-linking monomer, tetramethylenedimethacrylate (TMA). In preliminary trials, these cross-linked polyampholytes have exhibited satisfactory and reproducible properties as embedding materials for electron microscopy. The MA-DMA monomer mixture is miscible with water in all proportions. In recent years increasing attention has been given to water-soluble embedding media for various kinds of electron microscope investigations (2). Our limited

experience with these polyampholytes is therefore described in this paper in the hope that these systems will be of some general interest. Their utility in conjunction with ferritin-antibody staining techniques will be discussed in a separate communication (7).

MATERIALS AND METHODS

The monomer MA (Rohm and Haas Co., Philadelphia) was distilled at 10 mm pressure; the fraction boiling at 60°C was collected and stored at 4°C. DMA (Rohm and Haas) and TMA (Monomer-Polymer Laboratories, Philadelphia) tended to polymerize on vacuum distillation, and were therefore used as supplied by the manufacturers. The water content of the monomers was not controlled, and no difficulties appeared to arise as a result. Azodiisobutyronitrile (Eastman Organic Chemicals, Rochester, New York) was used as the polymerization catalyst.

When MA and DMA are mixed, considerable heat is evolved. To prevent any thermal polymerization on mixing, therefore, MA was added drop by drop, with stirring, to DMA (or to a DMA-TMA mixture) at 0°C to give the desired monomer composition. The catalyst, if desired in the mixture, was added last. Monomer mixtures were kept at 4°C and were used within 12 hours after mixing.

Small pieces of freshly excised tissue were fixed in 5 per cent glutaraldehyde (8) in 0.1 M phosphate buffer at pH 7.5 at 0°C for 2 hours. The tissue was then thoroughly washed with cold phosphate buffer, and left submerged in a known volume of buffer. To this was added a 1:1 molar mixture of MA and DMA in successive increments corresponding to 10 per cent, 20 per cent, 40 per cent, and 80 per cent monomers by volume. (The pH of a solution of the 1:1 monomer mixture in water is close to 7). The tissue was equilibrated at each step for 30 minutes at 4°C. It was then immersed in three successive batches of the 100 per cent monomer mixture over a period of 2 hours before being transferred to the embedding medium. The



FIGURE 1 Section of glutaraldehyde-fixed mouse pancreas embedded in the 2:1:0.33 MA:DMA:TMA cross-linked polyampholyte. No counterstaining was employed. Note the well-preserved endoplasmic reticulum, the mitochondrion with cristae (upper left), and the nucleus with granular interchromatinic substance. Magnification 19,000.

embedding medium we have used most often consists of the monomers MA, DMA, and TMA in the molar proportions 2:1:0.33 together with 0.025 per cent azodiisobutyronitrile. (The excess of MA was intended to provide an excess of negative charge on the polyampholyte for the ferritin-antibody experiments to be discussed elsewhere.) Embedding has also been successfully carried out in a monomer mixture with the molar proportions 1:1:0.25 containing 0.025 per cent catalyst. After soaking for 12 hours at 4°C in several changes of the embedding medium, the tissue samples were

transferred to freshly prepared embedding medium contained in a gelatin capsule.

Polymerization was induced by ultraviolet radiation from a Westinghouse Black Light Blue fluorescent lamp, F15T8/BLB, at 4°C. The capsules were housed in a box lined with aluminum foil. Polymerization was uniform and was essentially completed in 2 days; the samples were allowed to cure at 5°C for another day without radiation before they were sectioned.

Sectioning was carried out on a Porter-Blum microtome with glass knives. Because of the hy-

drophilic nature of the polymer, some changes in the usual sectioning procedure were found necessary. The glass knives, cut from $\frac{1}{4}$ -inch thick Pyrex sheet, were used with an included angle of between 35° to 40° . A slow rate of passage of specimen past the knife resulted in frequent wetting of the block and knife faces, and hence this rate was substantially increased. Water only was used in the trough. The sections were collected on 1000-mesh electrodeposited copper grids without a supporting film. They were not counterstained, but were examined directly in an Akashi Tronscope Model TRS-50 electron microscope.

RESULTS AND DISCUSSION

The glutaraldehyde-fixed, unstained section of mouse pancreas (Fig. 1) shows a preservation and clarity of ultrastructure at least as satisfactory as that obtained with glutaraldehyde-fixed pancreas embedded in Epon (8) or with formalin-fixed pancreas embedded in polyglycol methacrylate (2). The appearance of the endoplasmic reticulum with attached RNP particles, the elongated mitochondria with cristae, and the granular interchromatinic substance all attest to a satisfactory preservation of ultrastructure. Similar results were obtained with the 1:1 MA:DMA copolymer as with the 2:1. While it would be desirable to extend these studies to other types of tissue, it is very likely that the cross-linked polyampholytes will prove to be generally applicable as embedding materials.

Polyampholyte embedding may be particularly useful for the preservation of the molecular structures and biochemical activities of macromolecules in fixed tissue, as well as for ultrastructural preservation in general. Polyampholyte media, having to some extent the character of fused salts, are even more highly polar than other water-soluble embedding media, such as Aquon or glycol methacrylate (2), and should have therefore not only less tendency to extract non-polar substances from the tissue but also less tendency to disrupt the native structures of protein and other macromolecules (9). Furthermore, they share with other free-radical polymerization systems the property that they exhibit little if any direct chemical attack on protein and other macromolecules during the polymerization process. Thus, it has been shown that bacterial antigens (10) and tobacco mosaic virus protein (11,7) retain the capacity to bind their spe-

cific antibodies after embedding in a vinyl-type polymer. (By contrast, during epoxy or polyester embedding, proteins and other macromolecules should certainly be chemically modified (12), and quite possibly inactivated, by reaction with the monomers). It may therefore be possible to use various staining methods for enzyme localization (*cf.* references 13-15) *directly* on sections of fixed tissue embedded in a cross-linked polyampholyte.

SUMMARY

A new class of vinyl-type embedding polymers is described, the cross-linked polyampholytes, which is characterized by extensive ionic character. The polymers are studded with positive and negative ionic charges, with a small net charge, and in this respect resemble protein molecules. The copolymerization of the water-soluble monomers methacrylic acid and dimethylaminoethylmethacrylate, together with a cross-linking agent tetramethylenedimethacrylate, produces embedding polymers which have satisfactory properties for electron microscopy. In preliminary trials, they permit good preservation of ultrastructure. Their possible advantages as embedding systems are discussed.

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