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Epoxy resin as fixative during freeze-substitution

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Abstract

An alternative protocol for freeze-substitution is described. Araldite/Epon embedding medium (20% in acetone) is first used as a stabilizer (as e.g., OsO_4) and then as embedding medium. The major components of the Araldite/Epon resin formulation react with proteins and lipids and provide for an excellent preservation and reasonable visualisation of the ultrastructure. The ultrastructural appearance can be deliberately influenced with the standard freeze-substitution procedure [Van Harreveld, A., Crowell, J., 1964. Electron microscopy after rapid freezing on a metal surface and substitution fixation. Anat. Rec. 149, 381–386.] using OsO_4 as stabilizing agent by protocols which degrade cytoplasmic and membrane proteins. Epoxy stabilized and embedded samples may become an important tool to get information about the effects of different reagents and protocols used in freeze-substitution. We believe that an in-depth understanding of the procedures is required to correctly interpret images and to complement studies of dynamic processes by light microscopy with reliable, highly detailed ultrastructural information. The block face of epoxy stabilized samples after ultrathin sectioning is highly suited for the analysis of the ultrastructure by AFM.

Keywords: Epoxy resin; Fixative; Freeze-substitution; High-pressure freezing; Protein degradation; Structural preservation; TEM; AFM

1. Introduction

Most of our knowledge about cellular ultra structure has been obtained by transmission electron microscopy of ultrathin sections of biological material, chemically fixed with aldehyde, and OsO_4 followed by dehydration in graded series of organic solvents. The problems of the preparative procedures based on chemical fixation have been described and illustrated in numerous publications (Ameye et al., 2000; Hayat, 2000; Hunziker et al., 1984). They mainly originate from structural alterations of the cellular constituents due to the interaction with fixatives and dehydrating agents (conformational changes of proteins, partial hydrolysis of proteins, shrinkage, and dimensional changes due to the loss of membrane semi permeability, loss of lipids, limited time resolution for dynamic cellular events etc.). Techniques based on rapid cryoimmobilisation have been shown to overcome many of these problems.

Freeze-substitution is now the most widely used procedure to prepare cryoimmobilized biological samples for ultramicrotomy combining advantages of cryofixation with the ease of sectioning at room temperature. Generally, acetone containing 1–2% of OsO_4 is used to dehydrate and stabilize frozen biological materials. Freeze-substituted samples are then embedded into epoxy or acrylic resins. While, the former provide good structural preservation and identification, the latter (Lowicryl, LR-Gold) may be better suited for immunocytochemical experiments, since impregnation and UV-polymerisation can be performed at high subzero temperatures, eliminating the need for OsO_4 (Humbel and Mueller, 1986; Monaghan et al., 1998) and because there is a less intense interaction between proteins and acrylic resins (Kellenberger et al., 1987).

A great variety of substitution protocols are described in the literature. These procedures are difficult to compare as they are usually optimized for the solution of a specific problem and the substitution regimes (time, temperature) are often incompletely reported in the publications. In addition, the quality of freezing is most often unclear and can

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vary from the amorphous (vitrification) to a crystalline state with undetectable or barely visible segregation patterns due to ice crystal growth. Experience suggests that the final structural appearance depends on both freezing quality and substitution protocol. Substantial evidence has been provided e.g. by Studer et al. (1995).

At present it appears nearly impossible to achieve a constant quality of freezing, and vitrification rarely occurs except in very thin superficial layers or of objects that contain significant amounts of natural cryoprotectants. The effects of all steps (freezing quality, dehydration, and recrystallisation during freeze-substitution, stabilisation by fixatives, interaction with embedding resins and heavy metal stains) need to be understood to make full use of the potential of the rapid freezing-freeze-substitution concept and to properly interpret ultrastructural results at a high-resolution level. Descriptions of biological ultrastructure more closely related to the living state are important for complementing studies of dynamic events in living cells by fluorescent light microscopy. In some selected cases, cryosections can serve as a reference by which the influence of the steps of a particular freeze-substitution procedure can be characterized. True cryosections, however, are obtained only from vitrified material (Al-Amoudi et al., 2004; Michel et al., 1992) and cryosectioning of non pre-treated biological samples has remained very demanding despite some significant technical progress (Al-Amoudi et al., 2003). At present, it remains uncertain, whether cytological samples (cells and tissues up to approximately 100 μm thick) can ever be routinely vitrified to permit high-resolution investigation of the mutual interaction of cell organelles, of cell organelles and macromolecular components of the cytoplasm etc. by electron tomography of cryo-sections (Baumeister, 2002). Many relevant questions might be approached by high-pressure freezing, freeze-substitution, and subsequent embedding, if the effects of the steps involved in freeze-substitution and embedding were known more in detail.

In this paper, we want to show that the epoxy and anhydride groups, present in epoxy resin embedding formulations can be used as powerful stabilising agents during freeze-substitution. The chemical interaction of the constituents of the common epoxy resin formulations with biological material has been discussed by Causton (1985). The reactions of the components in a particular epoxy resin formulation with biological material are very complex. The most frequent reactions between epoxy resin and proteins occur at sites rich in nucleophilic substituents and end groups. Table 1 shows the most important reactions of the individual components in an epoxy resin formulation: the reactions of anhydride (cross-linker) and epoxy groups with protein.

We investigated a new procedure for freeze-substitution which is based on acetone and epoxy resin for stabilisation and embedding to design an optimized preparation protocol for cryoimmobilized biological material for the analysis of intracellular structures by AFM and to better understand the effects of the various interactions of epoxy and anhydride groups with cellular components.

Table 1
Reaction between nucleophiles present in proteins and reactive groups of the epoxy resin

Nucleophiles	Occurrence in proteins	Reaction group
Carboxyl	Glutamic acid, aspartic acid, chain ends	Epoxy
Phenol	Tyrosine	Epoxy, anhydrides
Amino	Lysine, chain ends	Epoxy, anhydrides
Imidazole	Histidine	Epoxy, anhydrides
Guanidyl	Arginine	Epoxy, anhydrides
Indole	Tryptophan	Epoxy, anhydrides
Amide	Glutamine, asparagines	Epoxy, anhydrides
Aliphatic hydroxyl	Serin, threonine	Epoxy, anhydrides
Sulphydryl	Cystine	Epoxy

2. Materials and methods

2.1. Chemical fixation

Adult *Caenorhabditis elegans* were prepared by classical fixation. Specimens were fixed in 0.7% glutaraldehyde, 0.7% OsO_4 in 10 mM Hepes buffer for 1 h. After washing in 10 mM Hepes buffer, the tails and the heads of the worms were cut and the material was post-fixed in 2% OsO_4 in 10 mM Hepes buffer for 3 h. Prior to dehydration in graded series of ethanol, the specimens were carefully rinsed with water. The dehydrated specimen were embedded in Araldite/Epon epoxy resin (Jorgensen et al., 1995).

2.2. High- pressure freezing

Adult *C. elegans*, a kind gift of Prof. M. Gotti, ETH Zürich, and Dr. I. Wakker, MPI Heidelberg, were high-pressure frozen in cellulose capillaries as described earlier (Hohenberg et al., 1994).

Antenna of the parasitic wasp *Cotesia glomerata* (Hymenoptera: Braconidae), a kind gift of Prof. Dorn, ETH Zurich, was isolated from the life wasp at 4 °C and than immediately frozen.

Life cat's mite *Otodectes cynotis* was mounted in aluminium platelets filled with hexadecane and immediately frozen.

The human lung fibroblast tissue was frozen using carbon coated sapphire discs as described earlier (Monaghan et al., 2003).

All freezing procedures were performed by a HPM 010 high-pressure freezer (Bal-Tec, Principality of Lichtenstein).

2.3. Freeze-substitution and embedding

Standard freeze-substitution was performed in acetone containing 2% OsO_4, The samples were kept at −90 °C, −60 °C and −30 °C for 8 h at each temperature, and finally warmed to 0 °C. The substitution medium was replaced by pure anhydrous acetone immediately after having reached 0 °C. Between the steps, the temperature was raised by 1 °C/min. After warming to room temperature, the samples were embedded in Araldite/Epon embedding mixture which was composed of 49% w/w Araldite/Epon stock solution, 49%