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PRESERVATION OF THE CELL-BIOMATERIAL INTERFACE AT THE ULTRASTRUCTURAL LEVEL

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Abstract

Studying the tissue-biomaterial interface at the ultrastructural level is not without problems. Dissolution of the biomaterial in one of the dehydration or embedding media causes holes and shatter during sectioning or dislodgement of the biomaterial.

The fine tuning of the hardness of both biomaterial and embedding medium, as well as the introduction of butyl-2,3-epoxypropylether as an intermediate between the dehydration series and the Epon resin, improving the impregnation, will solve many of the problems mentioned.

With this improved technique good results were obtained with materials ranging from teflon, poly(Lactic acid) and polyurethanes to tissue culture polystyrene. No holes, shatter or dislodgement of the biomaterial was observed.

Key Words: Biomaterials, Cell-biomaterial interface, Embedding, Epon resin, Transmission electron microscopy, butyl-2,3-epoxypropylether, impregnation, Tert-butyl glycidylether

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Introduction

Many different materials are either used as implants or as substrates for cell- or tissue culture. Such materials are referred to as biomaterials. The interface between the two is often damaged during the process of preparation for light or electron microscopy. The main problems are: the dissolution of the biomaterial in chemical substances of the dehydration or embedding series, leading to holes or shatter in the sections after ultramicrotomy and dislodgement of the biomaterial from the adjacent tissue. The extreme difference in hardness between the biomaterial, the tissue and the embedding medium is often the reason for sectioning difficulties.

Therefore a balanced choice has to be made for each biomaterial with regard to the embedding medium. For light microscopy the choice of embedding medium can be made, depending on the hardness of the biomaterial: teflon, silicon, rubber and other soft materials can best be embedded in paraffin, glycol methacrylate (GMA) (Gerrits and van Leeuwen, 1987) or Technovit 7100 (Gerrits and Smid, 1983). Materials of extreme hardness can only be embedded in Epon (Luft, 1961) or Epon araldite (Glauert, 1974). This way, no change in hardness is encountered by the knife during sectioning. If biomaterial samples are destined for transmission electron microscopy, the embedding has to be performed in epoxy resins as e.g., Epon (Luft, 1961), Epon-araldite (Glauert, 1974) or Spurr (Spurr, 1969). The aromatic epoxy resins (e.g., Araldite) are much more viscous than aliphatic resins (Epon, Spurr), and penetrate tissues slowly. The maximum sample size is thereby limited to approximately 2 mm. Additionally, the hardness of the epoxy resin can be adapted to the hardness of the biomaterial by changing the ratio between Epon and plasticizer and/or hardener.

A prerequisite for optimal sectioning however is a good impregnation with all the dehydration and embedding media (Blaauw et al., 1989).

The regular procedure for preparation of a biomaterial for transmission electron microscopy is dehydration in ethanol-propylene oxide according to the method described by Luft (1961). Most biomaterials, however, are dissolved or affected by propylene oxide. Omission of propylene oxide usually results in a poor embedding and subsequently poor

Table 1. Various biomaterials, their ultimate purpose, specifications and literature references. The novel embedding techniques were successfully applied on these materials.

Materials	Purpose	Specifications, References
Polyurethane	Vascular prosthesis	Van der Lei, 1985, 1987
Poly (L-lactic acid)	hollow fibers for controlled drug release bone plates	low (100,000) mol. weight Schakenraad et al., 1988; high (900,000) mol. weight Leenslag et al., 1987
Polytetrafluoroethylene (PTFE)	vascular prosthesis soft tissue patch for abdominal wall repair	Schakenraad, 1989 Simmermacher, 1990
Tissue culture polystyrene	fundamental research cell-polymer interactions	Schakenraad, 1987
Polyvinylfluoride	fundamental research cell-polymer interactions	Schakenraad, 1989
Polethylene terephthalate	tissue culture	van Wachem, 1989
fluorethylene propylene copolymer	fundamental research cell-polymer interactions	Schakenraad, 1986

sectioning, showing holes, shatter and dislodgement of the biomaterial (Fig. 1).

This paper reports on improved embedding procedure for biomaterials for transmission electron microscopy to preserve the tissue-biomaterial inter-face. The application of butyl-2,3-epoxy propylether in the dehydration series instead of propylene oxide enabled us to obtain good impregnations. The exchange with the Epon resin was optimal and sectioning did not result in holes and shatter. An additional advantage of this improved method is the possibility of processing large specimens (up to 3 mm) for transmission electron microscopy. Furthermore, the biomaterial-tissue interface remained intact, enabling us to study interfacial phenomena.

Materials and Methods

Various biomaterials (see Table 1) were harvested from various experimental animals, after different implantation times with excess surrounding tissue. The samples were fixed in 2% glutaraldehyde (phosphate or cacodylate buffered, pH 7.4) for 2 hours at 4°C. After fixation, the specimens were washed five times with the same buffer, for 5-10 minutes at 4°C. Post-fixation with 1% OsO_4 (phosphate or cacodylate buffered, pH 7.4) was carried out immediately after the washing procedure. The time needed for post-fixation depended on the size of the tissue and varied from 2 to 12 hours. An alternative post-fixative, used to enhance membrane contrast in the tissue, was: 1% OsO_4 + 1.5% $K_4Fe(CN)_6$ in the same buffer (de Bruyn, 1968; Hulstaert et al., 1983). After post-fixation, the specimens were washed five times for 10 minutes at 4°C in buffer, and in the last washing step we used aqua bidest instead of buffer. Subsequently, staining en bloc with 2% uranyl acetate in 10% ethanol was performed to achieve more contrast in the material.

Dehydration was performed in an ethanol series at 20°C: 15 minutes in 30%, 15 minutes in 50%, 15 minutes in 70% and four times 1 hour in 100% ethanol. Instead of propylene oxide, butyl-2,3-epoxypropylether was used as an intermediate between ethanol and Epon. Butyl-2,3-epoxypropylether (Merck) is also known as tert-butyl glycidyl ether (manufactured by Polysciences, Inc., Warrington, PA, USA). After dehydration, the specimens were submerged in butyl-2,3-epoxy- propylether two times for 30 minutes at room temperature. Subsequently, the specimens were processed in a mixture of butyl-2,3-epoxypropylether and Epon (1:1) overnight at room temperature. The next day the specimens were submerged in a mixture of butyl-2,3-epoxypropylether and Epon (1:3) for 24 hours at room temperature. Finally the specimens were placed in a rotating device under vacuum for 24 hours at 4°C in an Epon mixture without the catalyst to improve impregnation. The next day we added a fresh Epon mixture with catalyst (DMP 10) and rotated for 12 hours at 4°C under vacuum. Subsequently, part of the Epon mixture was removed until a 3 mm thick Epon layer covered the tissue, after which it was stored at a pressure of 100 mm Hg for 5 hours at 40°C. The specimens were than embedded in a freshly prepared Epon mixture and polymerized for 3 days at 65°C. For the hard biomaterials we used Epon 10-0 [52% Epon 812, 47% MNA (hardener) and 1% DMP 10], for the intermediate biomaterials Epon 5-5 [48% Epon 812,

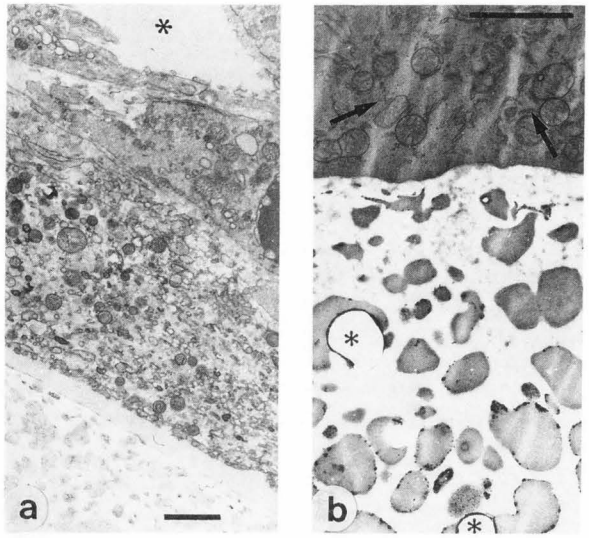

Fig. 1a shows dislodgement of tissue from the polymer due to differences in hardness, Fig. 1b shows holes (asterisk) and shatter (arrows) due to improper embedding. Bars = 4 μm.

23% DDSA (softener), 28% MNA and 1% DMP 10] and for the more flexible biomaterials we used Epon 3-7 (46% Epon 812, 35% DDSA, 18% MNA and 1% DMP 10). After polymerization, semi-thin sections (0.5-1.0 μm) were cut, using a Histo Diatome diamond knife, and stained with toluidine blue and basic fuchsin (Blaauw et al., 1987) to select the area of interest for electron microscopical investigation. Ultrathin sections (50-80 nm) were cut with a Diatome diamond knife and examined with a Akashi 002A transmission electron microscope.

Results

None of the biomaterials mentioned was affected by the butyl-2,3-epoxypropylether. The interface between biomaterial and tissue was completely intact. The interface of a poly (L-lactic acid) hollow fiber with subcutaneous fibrous tissue is shown in Fig. 2a. Notice the close alignment between cells and biomaterial. Fig. 2b shows a high magnification view of this interface; electron dense material can be observed between cell and solid (arrow). The contrast of the tissue is sufficient. If not, an additional staining en bloc with uranyl acetate and/or lead citrate can enhance the contrast. Figs. 3 and 4 illustrate cultured cells on solid surfaces. Fig. 3 shows a fibroblast on polytetrafluoroethylene (PTFE).

Fig. 4 shows an endothelial cell on poly(ethyleneterephthalate), a plastic used for tissue culture flasks and petri dishes. A close alignment at the interface can be observed without dislodgement caused by improper embedding or sectioning.

Discussion

Fine tuning of the hardness of biomaterial and embedding medium is a prerequisite for optimal sectioning and

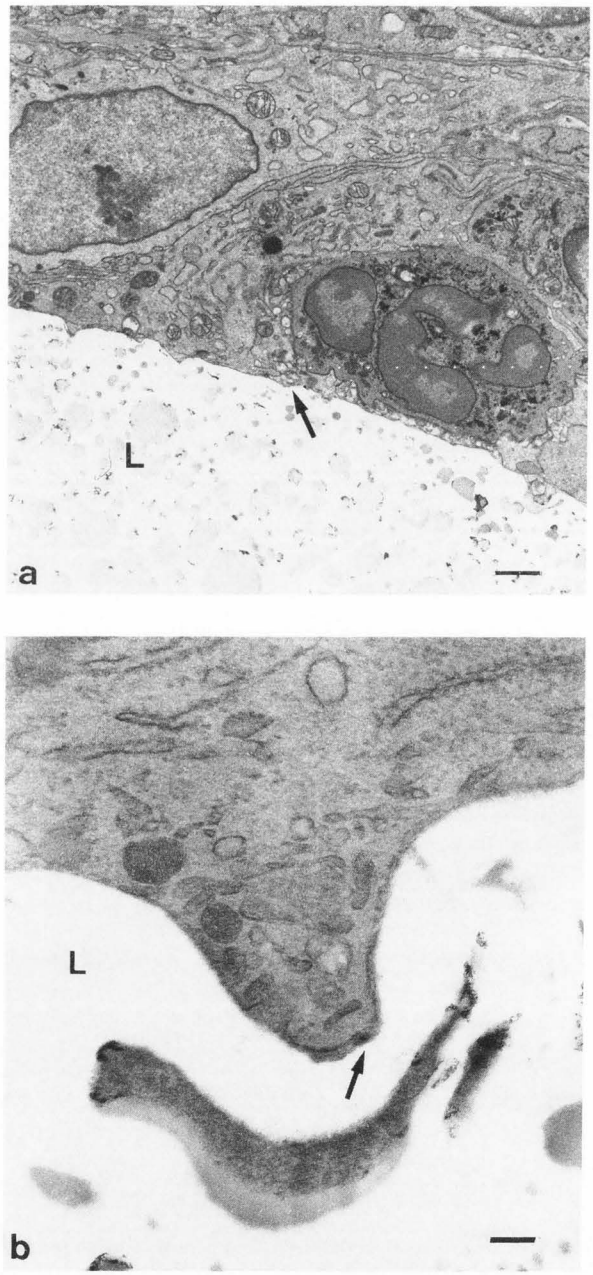

Figure. 2. Interface of a poly(L-lactic acid) hollow fiber with subcutaneous tissue. **(a)** Lactic acid (L) is a medium hard material, but it shows no signs of shatter or holes; close contact of tissue and biomaterial is obvious (arrow). **(b)** High magnification of the lactic-acid/tissue interface; electron dense material, probably of proteinaceous nature, can be observed between cell and solid (L).

Bars = 2.2 μm (in a) and 0.1 μm (in b).

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preservation of the interface.

The glass transition temperature is an indication for the brittleness at a certain temperature of resins and bio-

materials (Gerrits et al., 1990). If the glass transition temperatures of resin and biomaterial are comparable (and not over 35°C), less problems are expected during sectioning, at room temperature. It is therefore advised to choose the hardness of the embedding resin as close as possible to the hardness of the biomaterial.

The introduction of an intermediate between ethanol and epoxy resin can result in a better impregnation, in particular when the delta factor (solubility parameter) of epoxy resin and intermediate are similar (Gerrits et al., 1990). The delta factor of liquids can be calculated from the energy of evaporation (of molecular groups composing the repeating monomer) and the molar volume (Fedors, 1974). The delta factors of propylene oxide and butyl-2,3-epoxypropylether are almost identical and the intermediates are therefore interchangeable.

In addition, butyl-2,3-epoxypropylether is much more hydrophobic than propylene oxide and will therefore mix better with Epon, which is also rather hydrophobic.

With the embedding procedure, not using butyl-2,3-epoxypropylether, teflon-like materials are always disrupted from the tissue phase. Teflon is very hydrophobic (Schakenraad et al., 1986) and therefore most (hydrophylic) intermediates in the embedding series cannot properly impregnate this material. Apparently the butyl-2,3-epoxypropylether facilitates the impregnation, allowing close contact of Epon 812 with the solid materials. This completely successful impregnation is a prerequisite for proper sectioning and to avoid holes and shatter (Blaauw et al., 1989). Furthermore, the biomaterial remains adherent to the tissue impregnated with embedding medium.

Only polymer materials with high molecular weight or polymers which are completely inert can be embedded using the standard technique with propylene oxide.

To achieve optimal impregnation we have introduced very long impregnation times. Therefore the use of an intermediate between the ethanol series and Epon, that does not affect the polymeric material, is essential. The high vapour pressure of this intermediate might explain the improved impregnation.

We observed that, even in those cases where small amounts of butyl-2,3-epoxypropylether remained in the tissue during processing, the Epon blocks showed excellent sectioning properties after polymerization, possibly because the butyl-2,3-epoxypropylether is randomly incorporated in the polymerization reaction. In the transmission electron microscope the ultrathin sections showed no wrinkles, no holes, no shatter, and the adherence between the biomaterial and the tissue was excellent.

Conclusions

Biomaterial embedding in Epon 812 using butyl-2,3-epoxypropylether is applicable for large specimens with varying mechanical and chemical characteristics: from relatively soft and elastic to brittle and stiff, from completely bioinert to biodegradable. Especially these biodegradable materials are very susceptible for chemical agents. With a firm adhesion between tissue and biomaterial, no holes or shatter are observed in the ultrathin sections.

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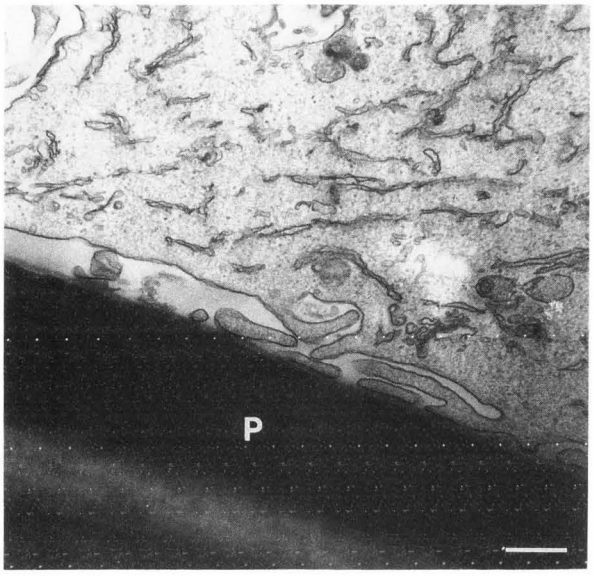

Figure 3. Interface of a cultured human skin fibroblast on polytetrafluoroethylene (P). Even though this cell has adhered to the PTFE with so called extracellular matrix contacts (distance > 100 nm.) the interface is not disrupted. The bar denotes 0.5 μm.

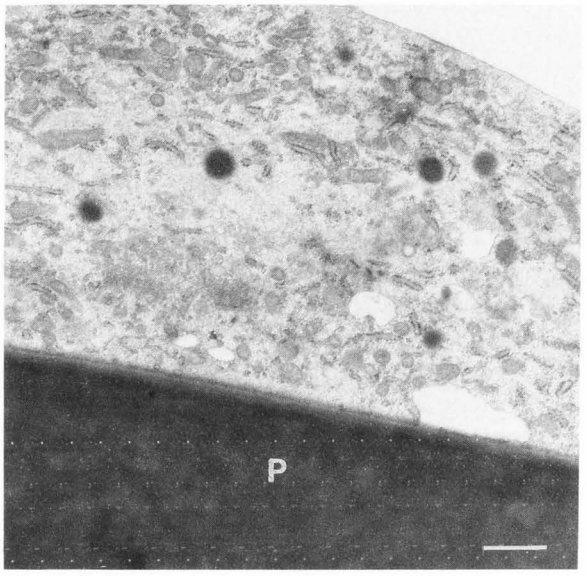

Figure 4. Interface of a cultured endothelial cell on poly-(ethyleneterephthalate) (P). Note the close approximation of the cell to the solid (distance 20-50 nm). The bar denotes 0.5 μm.

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Discussion with Reviewers

A.J. Wasserman: What biomaterials, if any, would be adversely affected by epoxypropylether?
Authors: Until now we have only encountered one material that was extremely affected by epoxypropylether: a biomaterial developed for degradable artificial skin [lysine diisocyanate-based poly (glycolide-co-E-caprolacton)], see Biomaterials **11** (1990) p. 291-295.

A.J. Wasserman: What would the deleterious effect consist of?
Authors: Solubilization of the polymer. We do note that if other biomaterials are exposed to 100% epoxypropylether for longer time intervals (> 36 hours) some effects might occur.

A.J. Wasserman: Are there any biomaterials that you have worked with that this method did not help to infiltrate with resin?
Authors: Biomaterials which are solid and very hard are poorly impregnated with this chemical. On the other hand, these materials can often be treated with propylene oxide without any problems.

A.J. Wasserman: What would happen if you did not place the tissue under 100 mm Hg pressure during the embedding procedure?
Authors: Air bubbles might be enclosed in the resin resulting in holes and/or dislodgement.

G. Pasquinelli: In the last years some compound biomaterials were introduced in the market, e.g., gelatin and albumin-coated Dacron in the cardiovascular field. Such materials are particularly difficult to be investigated at electron microscopy level. In fact, they obviously present different chemical and physical characteristics. What approach you recommend to study phenomena occurring at the interface in such materials.
Authors: Dacron (polyethylene therephthalate) can be embedded using propyleneoxide. Epoxypropylether will certainly be acceptable. Gelatin and albumin (provided they are

firmly fixed to the polymer) can (after cross-linking with glutaraldehyde) also be embedded in propylene-oxide. We do recommend to apply long incubation times with the different intermediates, since improper impregnation will certainly result in dislodgement at the interface.

A.R. Spurr: Is butyl-2,3-epoxypropylether compatible with the various epoxy resin embedding media in all proportions?
Authors: Butyl-2,3-epoxypropylether is compatible with Epon, Epon-araldite and Spurr resin. As an intermediate it is mixable in all proportions. Before sectioning however, it is important that the major part of the intermediate is exchanged by the resin, since large amounts of remaining butyl-2,3-epoxypropylether might impair sectioning quality.

A.R. Spurr: Why should the use of butyl-2,3-epoxypropyl-ether promote a better interface between tissue and various synthetic implant materials than the use of more conventional dehydration systems?
Authors: The addition of butyl-2,3-epoxypropylether will improve impregnation of the final resin, possibly by its high vapour pressure. It improves the adhesion of the resin to the biomaterial interface, even to relatively hydrophobic surfaces. The authors, however, do not consider butyl-2,3-epoxy-propylether a part of the dehydration series.

A.R. Spurr: The use of the term biomaterials gives the impression that they are directly of biological origin, whereas they are either plastics or synthetic materials. Wouldn't a term such as implant materials be more appropriate?
Authors: The term implant materials would certainly describe the purpose of these materials more adequately, however, the term biomaterials, which is generally accepted, describes the intended interaction of the material with biological tissues. Secondly, several biomaterials are indeed of biological origin, as e.g., collagen, catgut, cellulose and silk.