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A VERSATILE, LOW TOXICITY GLYCOL METHACRYLATE EMBEDDING MEDIUM FOR USE IN BIOLOGICAL RESEARCH, AND FOR RECOVERED BIOMATERIALS PROSTHESES

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Abstract

Methods for preparing standardized glycol methacrylate (GMA) based embedding media for use in light microscopy in a rational, precise and reproducible manner are described. The application of these procedures resulted in a versatile, low toxicity GMA embedding medium.

GMA embedding medium and resin blocks were tested utilizing a variety of physico-chemical techniques, namely: gas chromatography, determination of the maximum temperature reached during polymerization, the time taken to reach the maximum temperature, hardness testing, determination of the glass transition temperature, and measurement of the dimensional changes following section stretching and mounting at various temperatures. Data obtained from these techniques enabled a multi-purpose GMA embedding medium to be precisely specified.

The infiltration solution, as well as the accelerator solution, of this mixture contains fewer toxic components than comparable systems. The infiltration solution consists of GMA monomer and a non-toxic plasticizer (2-isopropoxy ethanol). The initiator is a 50% damped dibenzoyl peroxide, and the accelerator solution is composed of a mixture of polyethylene glycol 200 and a low concentration of a low toxicity tertiary amine (N,N,3,5-tetramethylaniline). Resin blocks obtained from this mixture are highly transparent, do not become coloured, and are easy to cut at 0.5-2.0 μm.

Various histological techniques, such as routine embedding of implanted biomaterials, histochemistry, enzyme histochemistry, and immunohistochemistry were carried out; the new GMA embedding medium proved to be applicable in all the techniques without laborious modifications.

Key Words: Glycol methacrylate; GMA; biomaterials; resin embedding; plasticizer; enzyme histochemistry; immunohistochemistry; toxicity.

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Introduction

2-Hydroxyethyl methacrylate, HEMA (also known as glycol methacrylate or GMA) is a widely used embedding medium in light microscopy. Embedding tissues in GMA enables the preparation of semi-thin sections (0.5-2.0 μm) of high quality with extremely well preserved morphology. These advantages of GMA over other embedding media have evoked a considerable interest in the use of such media for research on biomaterials, and in histo-technology.

Until recently preparing new, versatile methacrylate-type embedding media for light microscopical research has been an empirical activity. However, these infiltration and embedding media have to contain several fundamental components: monomer, plasticizer, initiator, and accelerator. Based on the pioneering work with GMA mixtures (Ruddell, 1967a, b; Sims, 1974), we have previously attempted to standardize the composition of GMA embedding media which consist of peroxides in combination with tertiary amines (Gerrits, 1987; Gerrits and van Leeuwen, 1985, 1987). Purity of the monomer; the relationship between various concentrations of dibenzoyl peroxide, N,N-dimethylaniline and inhibitors; and the maximum temperature and time of polymerization, were included in these studies. The effects of the physical properties of the GMA resin on histo-processing has also been investigated (Gerrits and van Leeuwen, 1984; Gerrits et al., 1987; Hanstede and Gerrits, 1983). Overall it was found that reliable and reproducible GMA embedding media could be prepared. However, their toxicity remained a major disadvantage. A logical next step was the replacement of the toxic components in these GMA media by less toxic ones. In this program the first step was an attempt to replace the toxic **plasticizers**, and subsequently to find substitutes for the toxic tertiary amine **accelerator solutions**.

Plasticizers

The addition of plasticizers to GMA media lowers the glass transition temperature (Tg) and hardness of the derived polymers. However, the chemical nature of the final polymer (Gächter and Müller, 1985) is not affected. Below their Tg, thermoplastic resins, such as GMA

and HPMA (Hydroxypropyl methacrylate), are relatively brittle and consequently, difficult to cut. The Tg of GMA is 55 °C, so addition of plasticizers is required to lower this value towards room temperature. Resin embedding media thus must contain sufficient plasticizer to achieve this.

We have developed a numerical procedure (parameters: molecular weight, melting point, solubility parameter, hydrophilicity/hydrophobicity, rat oral LD_{50}, and hazard information) to select less toxic plasticizers (Gerrits et al., 1990). A series of eight plasticizers was tested for their ability to replace 2-butoxyethanol and cyclohexanol in GMA embedding media. It was shown that the less toxic plasticizers 2-isopropoxyethanol and 2(2-ethoxyethoxy)ethanol can replace 2-butoxyethanol and cyclohexanol without reducing the quality of the results obtained.

In the present study we focussed on the application of 2-isopropoxyethanol (IP) as the plasticizer in GMA mixtures. IP permits the preservation of excellent morphological detail and the retention of moderate to high levels of enzyme activity (Gerrits et al., 1990). It can also be used for immunohistochemistry (unpublished observations).

Accelerator solutions

In GMA mixtures which make use of peroxides in combination with tertiary amines as initiator/accelerator systems, the commonly used N,N-dimethylaniline is another toxic component. We therefore wished to replace this toxic tertiary amine by less toxic amines. In this we were aided by the results of Bowen and Argentar (1971), in which various tertiary aromatic amine accelerators for methacrylate resins used in dental research were described. On the basis of this study, four commercially-available tertiary amines [N,N-dimethylaniline; N,N-dimethyl-p-toluidine; N,N,3,5-tetramethylaniline(N,N-dimethyl-sym-m-xylidine); and 4-tert-butyl-N,N-dimethylaniline] were selected for application to biomaterial research and histo-techniques.

Various physico-chemical procedures were used to determine the suitability of components and mixtures, and to fine-tune the properties of the ultimate GMA embedding medium for histo-technical purposes:

1. Gas chromatography was used for the analysis of GMA monomer samples, and to check the final composition of resin mixtures.

2. Measurements of maximum temperatures (T_{max}) reached during polymerization were performed, to determine the effects of exothermic heat.

3. The times of the maximum polymerization temperatures (tT_{max}) were determined to define acceptable processing times.

4. Ball indentation hardness and glass transition temperatures (Tg) were measured to determine the hardness of resin blocks, and to predict their cutting properties.

5. Dimensional changes of sections following stretching and mounting at various temperatures were measured, to predict the suitability of resins for morphometric analysis.

6. To assess the efficiency of the resins, test systems comprising various tissues and implanted biomaterials (e.g., polyurethanes) were processed and embedded for histochemistry, enzyme histochemistry, and immunohistochemistry.

Data derived from this battery of tests were compared with equivalant data from two widely used, GMA-based, commercial embedding media: JB-4, and Technovit 7100 - Historesin.

The aim of the present study was to develop a versatile, low toxicity GMA embedding medium, using dibenzoyl peroxide in combination with a less toxic tertiary amine as the initiator/accelerator system. The embedding medium had to be appropriate for the application of a broad spectrum of light microscopical techniques, and to be useful in the study of tissue-biomaterial interfaces.

Materials and Methods

Materials and Sources

Glycol methacrylate monomer (2-hydroxyethyl methacrylate, HEMA) was obtained from Röhm GmbH Chemische Fabrik, Darmstadt, Germany with the following specifications: HEMA > 99%, methacrylic acid < 0.05%, alkylene dimethacrylate < 0.15%, stabilized with 200 ppm hydroquinone monomethyl ether.

2-Isopropoxyethanol, IP (product no. 59670) and polyethylene glycol 200 (product no. 81150) were supplied by Fluka AG, Buchs, Switzerland.

Ethylene glycol dimethacrylate (product no. 818847) was from Merck-Schuchardt.

Dibenzoyl peroxide, from J.T. Baker, Chemical Co, San Francisco, USA (product no. 9104) was moistened with 20% H_2O (w/w).

Lucidol CH-50 (dibenzoyl peroxide damped with 50% dicyclohexyl phthalate) was from Akzo Chemie GmbH, Noury-Initiatoren, 5160 Duren, Germany.

N,N-dimethylaniline, DMA (product no. D14, 575-0); N,N-dimethyl-p-toluidine, DMPT (product no. D18,900-6); N,N,3,5-tetramethylaniline, DMSX (product no. 17,734-2) and 4-tert-butyl-N,N-dimethylaniline, DMBA (product no. 20,987-2) were purchased from Aldrich Chemie, Steinheim, Germany.

Technovit 7100 and Historesin were manufactured and supplied by Heraeus Kulzer GmbH, Friedrichsdorf, Germany.

The JB-4 embedding kit was obtained from Polysciences Limited, St. Goar, Germany.

Embedding moulds and aluminium block holders were purchased from DuPont Instruments, Biomedical Division, Newton, Connecticut, USA.

Sections were cut on a Reichert-Jung 2050 Supercut microtome, using a D-Knife with a tungsten carbide cutting edge (Leica Instruments GmbH, Nussloch, Germany).

Table 1. **Low Toxicity Embedding Medium**

Solution A (RES G20 Infiltration Solution)	
- Glycol methacrylate (200-300 ppm hydroquinone monomethyl ether)	90 ml (monomer)
- 2-Isopropoxyethanol	10 ml (plasticizer)
- Ethylene glycol dimethacrylate	0.4 ml (crosslinker)
- Lucidol CH-50 (dibenzoyl peroxide) (damped with 50% dicyclohexyl phthalate)	0.6 g (initiator)
Solution B (RES G20 Accelerator Solution)	
- Accelerators*	1 part
- Polyethylene glycol 200 (carrier for the accelerators)	30 parts by volume
Solution C (Embedding Solution)	
- Solution A : Solution B (v/v),	varied from 20:1 to 60:1

* N,N-dimethylaniline; N,N-dimethyl-p-toluidine; N,N,3,5-tetramethylaniline; 4-tert-butyl-N,N-dimethylaniline.

Methods

Preparation of GMA mixtures. Solution A, the infiltration solution (see Table 1): Low acid GMA samples (200-300 ppm hydroquinone monomethyl ether) were selected, and sufficient ethylene glycol dimethacrylate was added to bring the final concentration of this cross-linker to 0.3-0.5%. This crosslinker improves the stability of the sections in alcoholic and alkaline staining solutions, as described previously (Gerrits and van Leeuwen, 1987). 2-Isopropoxyethanol was added as plasticizer in a 10% (v/v) concentration in the GMA (Gerrits et al., 1990). A minimal content of dibenzoyl peroxide (0.3% w/v) was used as initiator (Gerrits and van Leeuwen, 1987). During the experiments, Solution A was used in a constant concentration and coded as RES G20.

Solution B, the accelerator solution (see Table 1): Accelerator solutions were made up of one of the tertiary aromatic amines, and polyethylene glycol 200 (plasticizer and a carrier for the accelerator), in volume ratios varying from 1:15 to 1:60.

Solution C, the embedding solution: Solution C was composed of mixtures of Solution A and Solution B, with volume ratios varying from 20:1 to 60:1. Solutions B and C were varied to provide a range of GMA embedding mixtures with differing T_{max} and tT_{max} values.

Gas chromatography. A Sigma 2000 gas chromatograph from Perkin Elmer was used to determine the concentrations of methacrylic acid, crosslinkers, plasticizers and inhibitors present. RES G20 infiltration solution A was compared with Technovit 7100 (Gerrits and Smid, 1983) and JB-4 (Polysciences 1976).

Determination of maximum polymerization temperatures (T_{max}) and the times of T_{max} (tT_{max}). A digital temperature recorder (Linseis, Germany, type LPD 12) equipped with Fe/Co thermo-elements was used to determine T_{max} and tT_{max}. Measurements were made in polyethylene moulds provided with aluminium blockholders, containing 2.5 ml resin at 4 °C or at 20 °C. In all experiments a rim of melted paraffin was poured around the blockholders to prevent inhibition of the polymerization by atmospheric oxygen.

Hardness testing. The hardness of resin blocks prepared using RES G20, Technovit 7100, and JB-4 was determined by means of ball indentation using a Zwick hardness tester, type 3106.1/01 according to DIN 53456. Data are given in N/mm^2.

Measurement of glass transition temperatures. The glass transition temperatures of RES G20, Technovit 7100, and JB-4 resins were measured with a Baehr dilatometer, type D 803.

Dimensional changes of sections during stretching and mounting. The stretching behaviour of sections of RES G20, Technovit 7100, and JB-4 was determined as follows: Five blocks of pure resin and five blocks of resin-embedded liver were sectioned dry with the microtome set at 2 μm. Sections were stretched (expanded) on distilled water, mounted on slides, and allow to dry at room temperature or at 60 °C on a hot plate. Five sections from each block were measured with a Wild M5 stereomicroscope and vernier calipers. The length of each section was measured in the cutting direction, and the width measured perpendicular to it. Mean values for five sections were used for further calculations.

Tissue fixation and routine embedding. Various tissues and implanted biomaterials, such as polyurethanes and poly L-lactic acids, were fixed by immersion in 4% (w/v) formaldehyde in 0.1 M phosphate buffer pH 7.4 for 12 hours at room temperature. Formaldehyde was prepared from paraformaldehyde according to Karnovsky (1965). After fixation, ascending concentrations of ethanol were used for dehydration. The following processing schedule was used: 70% (v/v) ethanol, 2 hours; two changes of 95% (v/v) ethanol, 2 hours each and absolute ethanol, 1 hour. Subsequently the specimens were pre-infiltrated for 2 hours in a solution consisting of equal volumes of absolute ethanol and Solution A (Table 1). Infiltration was then carried out in Solution A for 12-48 hours. Depending on the size of the tissue specimens, infiltration times were prolonged up to 3-4 days. Following infiltration, the tissue samples were placed in the embedding moulds containing the embedding Solution C (i.e., infiltration Solution A to which the accelerator solution B had been added, see Table 1). It is recommended to bring the tissues in contact with Solution C for 10 minutes prior to placing them in the embedding moulds.

Block holders were placed into the embedding moulds, and melted paraffin (60 °C) was poured around

the block holders. Dehydration, infiltration and embedding were carried out at room temperature. Following polymerization the resin blocks were stored at room temperature. Two μm thick sections were cut dry under controlled, standardized conditions in a temperature controlled room (23 °C), at 60% relative humidity. They were stretched on distilled water, mounted on slides and dried at 60 °C for 10 minutes.

Enzyme histochemistry. Tissue slices, 2-4 mm thick, were fixed in 2% formaldehyde, in 0.02 M phosphate buffer pH 7.4 containing 5% sucrose for 4 hours at 4 °C (Hantschick et al., 1988). Dehydration was carried out with ascending concentrations of ethanol using the following processing schedule: 70% (v/v) ethanol, 1 hour; 95% (v/v) ethanol, 1 hour; followed by absolute ethanol, 2 hours. Infiltration with RES G20 Solution A was carried out overnight. Tissue processing from fixation to infiltration was performed at 4 °C. During fixation and processing the tissue samples were gently agitated on a roll action mixer. To disperse heat liberated during polymerization, embedding moulds were placed on crushed ice in a refrigerator. Sections were cut dry with the microtome set at 2 μm, stretched on distilled water, mounted on slides, and air dried at room temperature (20 °C) overnight. Sections were cut under the standardized conditions described above.

Immunohistochemistry. Tissue specimens of human tonsils, 1 mm thick, were fixed in 2% formaldehyde in phosphate buffered normal saline (PBS) for 3 hours at 4 °C. Subsequently the tissue was washed in a 0.02 M phosphate buffer containing 6% sucrose, overnight at 4 °C. Dehydration was in absolute acetone at 4 °C for 1 hour. The acetone was repeatedly changed until the solution remained clear. Following dehydration the tissues were infiltrated with RES G20 for 6-8 hours at 4 °C. During the entire procedure, the tissues were gently rotated. The resin was polymerized overnight at 4 °C on crushed ice.

Staining procedures

Sections were stained using a panel of staining procedures: Hematoxylin and Eosin (H&E); Toluidine Blue; periodic acid Schiff (PAS); Jones' periodic acid methenamine silver (PAMS); Perls' Prussian Blue reaction; Giemsa; and a modified reticulin stain (Gerrits and Suurmeijer, 1991). Sudan Black B (Hoeksma et al., 1988) and Toluidine Blue were used to visualize interfaces between implanted biomaterials and tissues.

Enzyme histochemistry. Alkaline phosphatase was demonstrated according to Burstone (1962); acid phosphatase according to Barka and Anderson (1963); and non-specific esterases (i.e. alpha-naphthyl acetate esterase) according to Bancroft and Stevens (1982).

Immunohistochemistry. Staining procedures were performed according to van Goor et al., (1988), with slight modifications. Sections were pretreated with 0.01 % (w/v) trypsin in 0.17 % $CaCl_2.2H_2O$ for 5 to 10 minutes at 37 °C, washed in PBS (pH 7.4) for 7 minutes and incubated in the primary antibody for 2 hours at 37 °C. After another wash in PBS, endogenous peroxidase was blocked with 0.06% (v/v) H_2O_2 in PBS for 30 minutes at room temperature. Thereafter the sections were washed again in PBS for 7 minutes. The second, peroxidase conjugated antibody was applied in an appropriate concentration for 1 hour at room temperature, followed by a final wash in PBS. Peroxidase activity was localized using diaminobenzidine tetrahydrochloride dihydrate (DAB) for 12 minutes.

Sections were counterstained with Hematoxylin for 15 seconds, and washed in running tap water for 10 minutes until blue. A series of monoclonal antibodies, including reagents directed against T cells, subset T cells, B cells, and macrophages was tested. This series contained 35 reagents.

Results

Preparation of GMA mixtures

We only used GMA samples which had a polymerization inhibitor concentration in the range of 200-300 ppm, as assessed by gas chromatography. For these GMA samples the pH of 10% solutions of the monomer in distilled water was measured (Cole, 1984). Samples with pH values < 4 proved to be unsuitable, due to the presence of excess methacrylic acid.

The composition of the final RES G20 infiltration Solution A was adjusted with ethylene glycol dimethacrylate (EDMA) to approximately 0.4 vol% as determined by gas chromatography. This was approximately 0.3-0.4 ml of EDMA in 100 ml RES G20.

Accelerators

In initial experiments, four tertiary aromatic amines were investigated, with the following results:

1. N,N-Dimethylaniline (DMA) was the least effective of the four accelerators tested. It could be used in a concentration of 20-35 mmol. Blocks obtained with DMA were slightly yellowish, but turned into a deep brown colour when exposed to daylight.

2. N,N,3,5-Tetramethylaniline (DMSX) was an effective accelerator. It produced the lightest coloured resin blocks, which were the most colour stable when exposed to daylight. RES G20/DMSX blocks had only a slightly yellowish appearance. DMSX could be used at low concentrations, in the range 6-12 mM.

3. N,N-Dimethyl-p-toluidine (DMPT) can also replace N,N-dimethylaniline as the accelerator; and can be used in considerably lower concentrations (8-10 mmol) than DMA, to obtain comparable polymerization times. However, blocks obtained with DMPT showed considerable yellowing when exposed to daylight.

4. 4-tert-butyl-N,N-Dimethylaniline (DMBA) proved to be poorly miscible with PEG 200, and partly precipitated. Nevertheless the mixture could be used after filtration. DMBA repeatedly showed less consistent embedding results than those obtained with DMSX. Tissues frequently showed inhomogeneous, partially polymerized regions. Consequently, polymerization times were difficult to control and to standardize. A further disadvantage of DMBA is its high cost.

Table 2. **Gas Chromatographic Analysis of Solutions A of RES G20, Technovit 7100, and JB-4**

Resin Mixture Solution A.	MA (%)	EDMA (%)	Plasticizer (%)	Inhibitor (ppm)
RES G20	0.02	0.4	11 (IP)	305 HQME
Technovit 7100	0.02	-	10 (PEG 400)	290 HQME
JB-4	0.02	-	15 (BE)	280 HQME

Abbreviations: MA, methacrylic acid; EDMA, ethylene glycol dimethacrylate; IP, 2-isopropoxyethanol; PEG 400, poly-ethylene glycol 400; BE, 2-butoxyethanol; HQME, hydroquinone monomethylether. % (v/v).

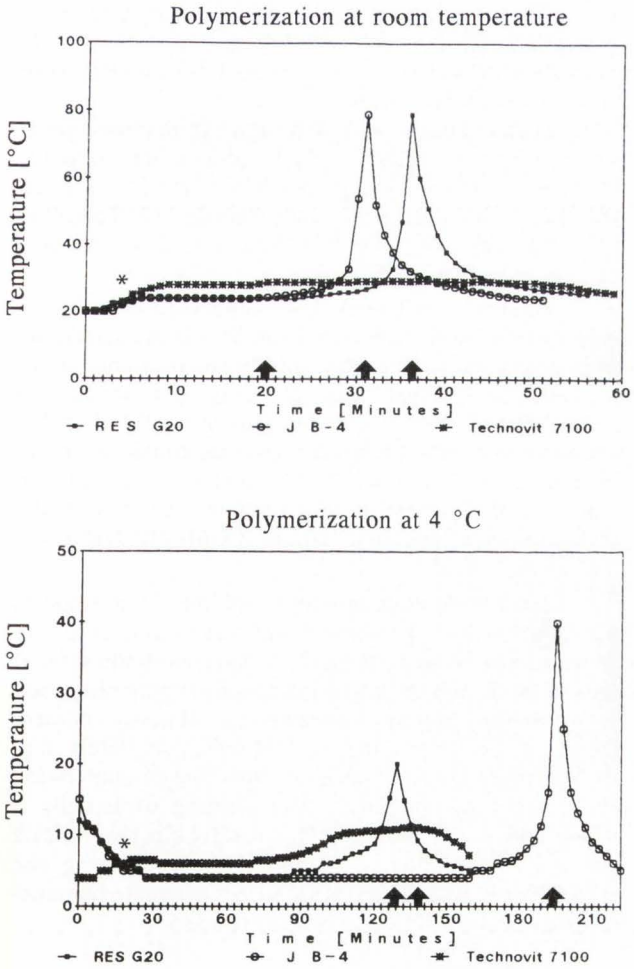

Figure 1. Representative curves showing changes in temperature during the course of polymerization at 20 °C (**A**) and at 4 °C (**B**) of RES G20/DMSX, Technovit 7100 and JB-4. RES G20 was used in a ratio Solution A to Solution B of 40 : 1; Technovit 7100, 15 : 1; and JB-4, 30 : 1. ↑ Maximum temperatures (T_{max}) reached during polymerization, and the times of the maximum polymerization temperatures (tT_{max}). * The moment melted paraffin was poured around the blockholders.

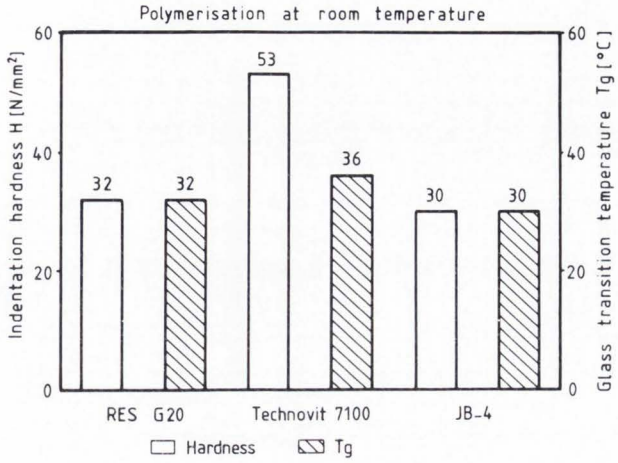

Figure 2. Glass transition temperatures and hardness of RES G20/DMSX, Technovit 7100 and JB-4 respectively. The composition of the embedding media was similar to those in Fig. 1.

Gas chromatography

Infiltration Solutions A of RES G20, Technovit 7100 and JB-4 were analyzed by gas chromatography (Table 2). All mixtures contained low concentrations of methacrylic acid (MA). In addition, RES G20 contained a minimal concentration EDMA, in contrast with Technovit 7100 and JB-4. RES G20 contained 2-isopropoxyethanol as plasticizer, whereas Technovit 7100 contained polyethylene glycol 400, and JB-4 contained 2-butoxyethanol.

Maximum temperatures (T_{max}) and the times of T_{max} (tT_{max})

Figures 1A and 1B show representative maximum polymerization temperatures (T_{max}) and the times of T_{max} (tT_{max}) of RES G20/DMSX, Technovit 7100, and JB-4 at 4 °C and 20 °C. At ambient temperatures of 20 °C, the T_{max} values of RES G20/DMSX and JB-4 were identical, the tT_{max} of JB-4 being reached about 5 minutes earlier (Figure 1A). The course of polymerization of Technovit 7100 is significantly different from that of RES G20/DMSX and JB-4.

At 4 °C a T_{max} of 20 °C for G20/DMSX and of 40 °C for JB-4 was recorded. The occurrence of tT_{max} of RES G20/DMSX was about 70 minutes earlier (Figure 1B).

Glass transition temperatures and hardness

Results from both Tg and ball indentation measurements are presented in Figure 2. The data for RES G20 and JB-4 were almost identical. Technovit 7100 blocks have a higher glass transition temperature, and as a consequence, are harder at room temperature. It was observed that varying the ratios between Solutions A and B in the RES G20 system significantly influenced the Tg, and consequently the final hardness, of the resin

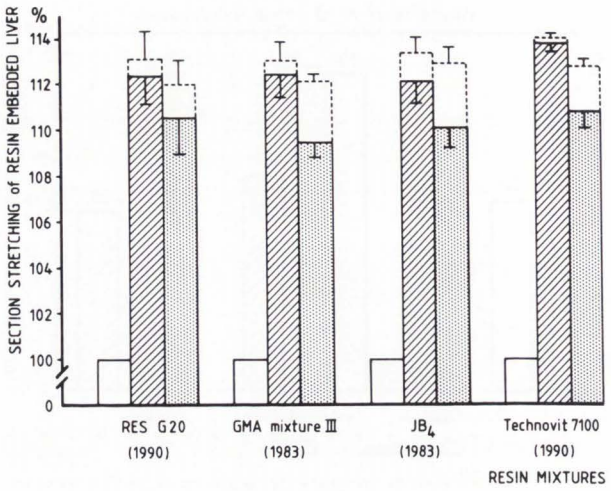

Figure 3. Effects of linear section stretching of 2 μm sections of RES G20/DMSX-embedded pig liver; GMA mixture III (Hanstede and Gerrits 1983); JB-4; and Technovit 7100, when mounted at 20 °C or 60 °C. Blank columns represent the size of resin-embedded tissue prior to cutting. Striped columns represent linear stretch in the cutting direction at 20 °C and stippled columns stretch at 60 °C. Dotted lines indicate the corresponding width of the sections. Vertical bars represent 1 $\pm$ standard deviation.

blocks. In addition, variations in polymerization times were noted. In short, low concentrations of Solution B (1:40/60) delayed the polymerization, but increased the Tg. With regard to high concentrations of Solution B (1:20/30) the reverse took place.

Tg measurements of our empirically composed GMA mixtures revealed that the Tg's of these mixtures are slightly above room temperature.

Microtomy

The use of DMSX as the accelerator for the polymerization of RES G20 did not influence the cutting properties of the final blocks, which resembled those of JB-4 blocks. The cutting properties of Technovit 7100 were the best of the embedding media tested.

Colour stability

DMSX, in combination with RES G20 mixtures, resulted in blocks of the lightest colour and of the highest colour stability. Resin blocks containing DMBA and DMPT had a lower colour stability, and blocks with DMA developed a pronounced deep brown colour.

Dimensional changes during stretching and mounting

In preliminary experiments we determined the stretching behaviour of tissue-free GMA sections from blocks prepared from various commercial GMA samples. Differences in stretching behaviour varied from 9-17% (linear) when stretched at 60 °C, and 12-20% at 20 °C. Tissue-free sections from RES G20 stretched about 12% at 60 °C, and 15% at 20 °C.

A comparison between sections of RES G20-embedded liver and those from 3 different GMA mixtures (GMA mixture III, data from 1983; JB-4, data from 1983; and Technovit 7100, data from 1991) is presented in Figure 3.

Routine staining, histochemistry, enzyme histochemistry, and immunohistochemistry

Routine staining and histochemistry. Semi-thin sections of tissues embedded in RES G20/DMSX are of excellent quality, and morphological detail is well preserved (Figure 4A). HE (Hematoxylin and Eosin), Toluidine Blue, Sudan Black B, PAS, PAMS, Perl's Prussian Blue, Giemsa, and a modified reticulin stain are applicable. For most stains only minor modifications in staining periods were needed.

RES G20/DMSX sections were stable in alcoholic and alkaline staining solutions and did not exhibit mini-folds following Hematoxylin and Eosin staining. In contrast to RES G20, sections from JB-4 showed these typical mini-folds artifacts.

Sudan black B showed a high affinity for hydrophobic polymers, such as poly L-lactic acids and polyurethanes. Implanted polymeric biomaterials stained with Sudan Black B, and counterstained with Toluidine Blue, showed a sharp contrast between tissue and biomaterial (see Figure 4B).

Enzyme histochemistry. RES G20/DMSX embedding enabled the demonstration of enzyme activity in a wide variety of tissue sites. Moderate to intense staining could be achieved after relatively short incubation times (1-2 hours). Alkaline phosphatase, acid phosphatase and alpha-naphthyl acetate esterase could be clearly demonstrated (Figure 4C). The results were equal in quality to those from previous studies (Gerrits et al., 1990), in which the combination of GMA/IP/DMA was extensively tested.

Immunohistochemistry. Of the 35 antibodies tested, about 75% performed well (van Goor et al., in preparation). Among these 75% were antibodies for B cells, T cells, and macrophages which are generally used in routine pathology laboratories. Twenty percent showed moderate staining, and 5% were completely negative. Figure 4D shows an example of the demonstration of an anti-IgM antibody. The staining of B cells is strong and well localized. Our results clearly indicate that the combination of careful tissue processing and embedding in RES G20/DMSX allows adequate immuno-histo-labelling in resin embedded tissue.

Discussion

Preparation of GMA mixtures

The selection of suitable GMA monomer samples proved to be crucial. Thus a low polymerization inhibitor concentration was needed, to permit standardization of polymerization conditions. A low methacrylic acid content was needed to avoid artifactual background staining of resin sections with basic dyes.

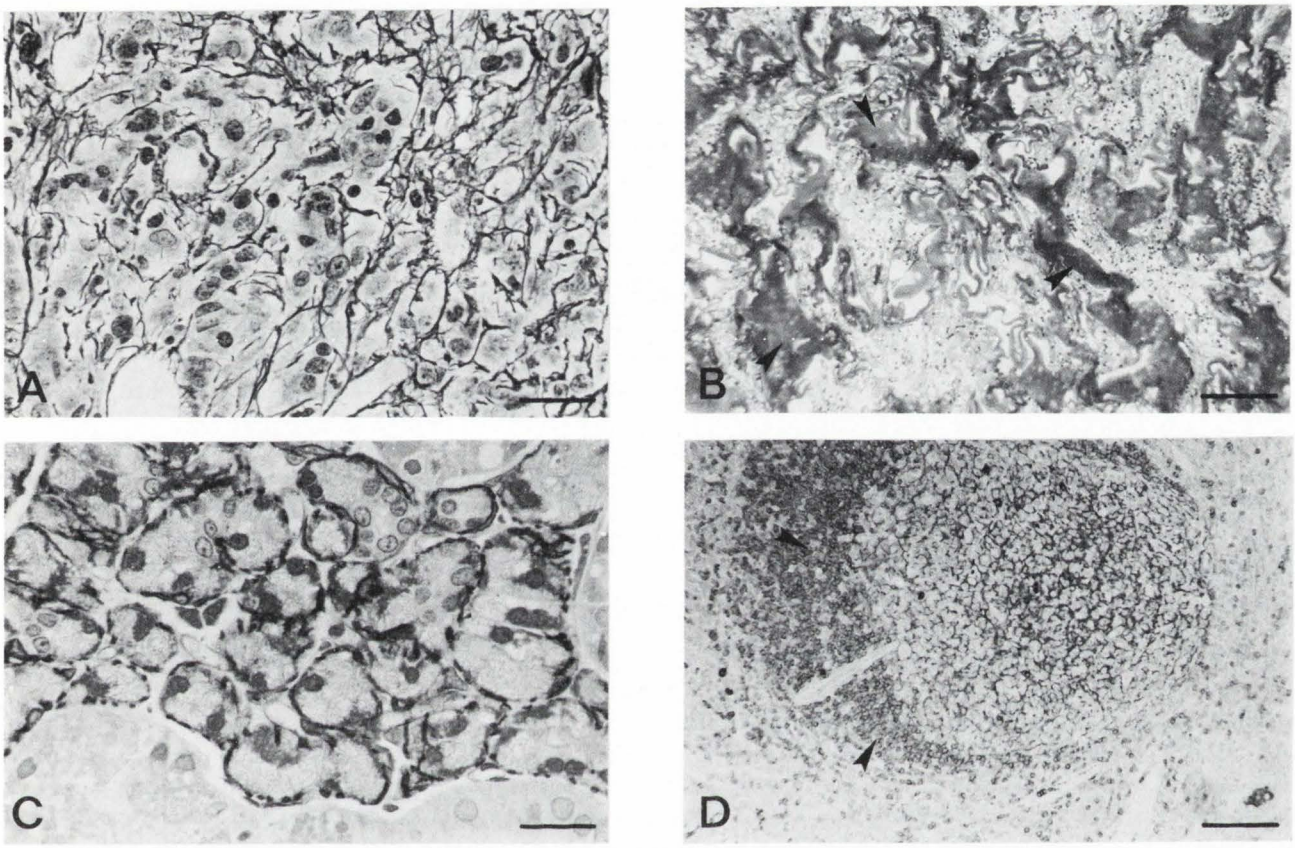

Figure 4. Semi-thin sections, 2 μm thick, of tissues embedded in RES G20. **(A)** Human bone marrow in iliac crest biopsy. Modified reticulin method. Bar = 25 μm. Figure kindly supplied by A.J.H Suurmeyer. **(B)** Polyurethane implant (➤) in cartilage defect stained with Sudan Black B, counterstained with Toluidine Blue. Bar = 100 μm. Figure kindly supplied by J. Klompmaker. **(C)** Rat submandibular gland. Alkaline phosphatase activity around myoepithelial cells. Alkaline phosphatase according to Burstone, counterstained with Neutral Red. Bar = 25 μm. **(D)** Human tonsil. Marginal zone B Cells (➤) stained with a monoclonal antibody directed against IgM, and counterstained with Hematoxylin. Bar = 100 μm.

Regarding other components of the embedding mixture, using approximately 0.4 vol% EDMA cross-linker in infiltration Solution A prevented wrinkles and mini-folds occurring when resin sections were exposed to alcoholic staining solutions or processing fluids. Use of N,N-3,5-tetramethylaniline gave the most transparent and colour stable resin, lowest toxicity, and the most even polymerization.

Solubility and toxicity of aromatic tertiary amines

The use of numerical structure parameters to guide selection of optimal compounds is well illustrated by this study. Thus in homologues of N,N-dimethylaniline, substitution of $-CH_3$ groups into the aromatic ring increases the hydrophobicity of the amine by nearly one Log P unit per methyl group [Log P (the logarithm of the partition coefficient of a compound between water and octanol-1) is a measure of the hydrophobic/hydrophilic character of a compound (Hansch et al., 1973)]. In general the higher the Log P value the less soluble a compound is in water.

In keeping with this, the more $-CH_3$ substituents the less soluble was the tertiary amine in the carrier (PEG 200). Indeed DMBA was difficult to mix with PEG 200.

Toxicities may be partly related to a compound's hydrophobicity (Dearfield et al., 1989). Hence in the present case, toxicity is expected to follow the sequence DMA > DMPT > DMSX > DMBA.

In addition to this effect, the concentration of the DMSX-containing solutions are significantly lower than those present in DMA-containing solutions. This further decreases the toxic properties of the DMSX accelerator solution. We were not able to collect relevant data on chemical safety, and for that reason must regard DMSX as an irritant (see Aldrich catalogue 1990-1991).

Temperature-time relationships during polymerization

The time of the maximum polymerization (tT_{max}) is possibly dependent on the aromatic tertiary amine used as the accelerator (Bowen and Argentar, 1971). These authors showed that highly active tertiary amines

were typically those with electron-donating substituents [e.g., $-CH_3$, $-C(CH_3)_3$] in the aromatic ring. It was also found that the difference in tT_{max} of DMSX and DMPT was statistically significant. A comparison between DMSX and DMA revealed that DMSX hardened a standard dimethacrylate mixture nearly 5 times faster than did DMA. DMBA was comparable with DMSX. When used in the standard RES G20 Solution A as a standard mixture, these components gave analogous results.

Glass transition temperatures and hardness

The glass transition temperatures of all media investigated, that is JB-4, Technovit 7100, and RES G20, are slightly above room temperature. Consequently while these resins are all in their rubbery state, they are nevertheless unlikely to be too soft. This suggests a rule of thumb: the optimal Tg for resin blocks is in the range 30-40 °C.

The hardness data is intriguing. The slightly elevated Tg of Technovit 7100 is reflected in the relatively harder character of this resin, that is 53 N/mm^2, compared with 30-32 N/mm^2 for the other resins. However it has previously been shown that the surface hardness of Technovit 7100 has the lower value of 39 N/mm^2 (Gerrits and van Leeuwen, 1984). Perhaps it is this surface layer of softer resin which accounts for the unusual capability of Technovit 7100 to produce ribbons of sections.

Microtomy

In earlier studies (Gerrits et al., 1990) we extensively tested a GMA/2-isopropoxyethanol system in combination with DMA. The sectioning properties of these blocks were similar to blocks resulting from standard mixtures. In fact the substitution of DMSX as accelerator in the present study made no difference to the cutting properties of the final resin block, which resembled JB-4.

Implanted polymeric biomaterials are difficult to section well. However if the Tg of these biomaterials is known, then a suitable GMA mixture can be chosen on rational grounds, because the Tg determines the brittleness/elasticity of resins and of biomaterials. If the Tg values of the resin embedding medium and of the biomaterial are similar, fewer problems should arise during sectioning (Schakenraad et al., 1990).

Colour stability of resin blocks

Bowen and Argentar (1971) found that colour stability was influenced more by the substituents on the aromatic ring than by those on the nitrogen atom. Resin mixtures with DMBA as the accelerator were the lightest in colour, and were most colour stable. Contrary to these findings, in the present investigation, it was DMSX which gave lighter and more colour stable resin blocks than did DMBA.

Dimensional changes during stretching and mounting

The physical properties of resin mixtures influence dimensional changes in tissues during the stretching of sections on water and mounting on glass slides at various temperatures. Significant differences in section stretching have been observed among methacrylates and methacrylate mixtures (Gerrits et al., 1987).

In the present study, the stretching of resin-embedded tissue sections usually followed that of pure resin, especially at room temperature. However, data from embedded tissues showed somewhat more variation. The data from RES G20 did not significantly differ from data derived from previous experiments (Gerrits and van Leeuwen, 1984; Gerrits et al., 1987; Hanstede and Gerrits, 1983). Data obtained from section stretching are important physical parameters to check the suitability of new GMA mixtures. Full implications of the results of section stretching have been described elsewhere (Gerrits et al., 1991).

Sectioning of GMA-embedded biomaterials can give rise to some specific problems. Thus artifacts such as wrinkles at the resin-biomaterial interface may occur. These artifacts are most pronounced in the least elastic biomaterials; while highly elastic biomaterials follow the section expansion during stretching and mounting, as do soft tissues. A possible way to avoid or reduce this problem is to use methacrylates, which are less elastic than pure GMA, e.g., 2-hydroxypropyl methacrylate (see also Gerrits et al., 1987). The use of low-elasticity GMA mixtures is another possible tactic. The addition of small amounts of methacrylates having high glass transition temperatures (e.g., methyl methacrylate, Tg 105 °C; isobornyl methacrylate, Tg 110 °C), to the standard Solution A is an example of this approach.

Staining RES G20/DMSX embedded tissue sections

Semi-thin RES G20/DMSX embedded tissue sections routinely show good morphology. A wide range of histological and histochemical staining methods can be applied to such sections. In addition biomaterials can be selectively stained.

Many routine enzyme histochemical and immunohistochemical procedures were also found to be applicable. However various adaptations may be required. These may involve merely the embedding step, or other histo-processing stages in addition.

Thus fixation fluids may need to be modified. Examples of this include lowering formaldehyde concentrations, and shortening of fixation time. Processing may also be altered: processing periods may be reduced, and processing may be carried out in the cold.

With specific regard to immunohistochemistry, it is advisable to use cold acetone instead of ethanol as the dehydration medium. To limit loss of enzyme activity or antigenicity due to exothermic heat, polymerization should be carried out on crushed ice. Measurement of the maximum temperatures reached during polymerization gives a clear indication of the heat released. Peak temperatures above 40 °C are harmful to enzymes and antigenic sites. However, it has to be stressed that even at low polymerization temperatures, use of barbituric acid derivatives/chloride ions as the initiator/accelerator system, present in Technovit 7100 and Historesin, yields inconsistent results with immunohistochemical tech-

niques. These observations are in agreement with those of Casey and Beckstead (1990). These authors also found that JB-4 gave more consistent results than did Historesin. A possible explanation might be that the separate components of this type of polymerization system interfere with antigenicity. Evidence for this statement was given by Hand (1988). The accelerator Solution B of Historesin, which contains the barbituric acid derivative, completely inhibited lactase and sucrase activity in frozen sections. Which components actually diminish antigenicity is still unknown and needs further elucidation. Unfortunately the JB-4 embedding kit contains a toxic irritant (2-butoxyethanol) as plasticizer, and a highly toxic irritant (N,N-dimethylaniline) accelerator.

When using the less toxic RES G20/DMSX mixture, we consistently demonstrated high levels of enzyme activity, as well as a broad spectrum of antigens.

Conclusions

1 - By studying a variety of physical parameters (namely dimensional changes, hardness, purity, Tg, T_{max}, tT_{max}) a versatile, low toxicity GMA embedding medium was formulated. This medium termed, RES G20/DMSX, utilizes dibenzoyl peroxide in combination with a tertiary aromatic amine as the initiator/accelerator system.

2 - The RES G20/DMSX embedding medium can be standardized and continuously controlled by means of a combination of physico-chemical measurements and histological procedures.

3 - The use of N,N,3,5-tetramethylaniline (DMSX) as accelerator yields GMA blocks that show minimal colouration, and optimal colour stability.

4 - GMA embedding media such as RES G20/DMSX are systems of choice for study of tissue-biomaterial interfaces, and permit the application of histochemical, enzyme histochemical and immunohistochemical techniques.

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Discussion with Reviewers

A.R. Spurr: The tests on toxicity focused on plasticizers, but RES G20 contains many other compounds. Apparently they are mainly from industrial sources and possibly in need of standardization or refinement. Don't you think there is too much uncertainty on toxicity for many of the constituents to make claims of low toxicity?
Reviewer II: The authors claim that the replacement of conventional plasticizer by 2-isopropoxyethanol and the accelerator amine by N,N,3,5-tetramethyl aniline results in a composition of lower toxicity. This may be so but the matter would be more convincing if toxicity data with LD_{50} or similar values were given to support the claim.
Authors: Water-miscible resins, such as GMA, are now widely used as embedding media for tissues in light microscopy. However, the major part of these mixtures contain toxic components. We have made attempts to replace toxic components, such as plasticizers and accelerators, with components of lower toxicity wherever possible. Convincing data which demonstrate that the degree of toxicity of 2-isopropoxyethanol is remarkably less than 2-butoxyethanol have been reported by NI Sax (in: Dangerous properties of industrial materials. 6th edn., Van Nostrand Reinhold, New York, 1984). The following comparable toxicity data were reported:

2-Butoxyethanol

oral rat	LD_{50}:	1480 mg/kg
inhalation rat	LCLo:	500 ppm/4H
skin rabbit	LD_{50}:	490 mg/kg
inhalation mouse	LC_{50}:	700 ppm

2-isopropoxyethanol

oral rat	LD_{50}:	5660 mg/kg
inhalation rat	LCLo:	4000 ppm/4H
skin rabbit	LD_{50}:	1600 mg/kg
inhalation mouse	LC_{50}:	1930 ppm

Concerning the toxicity of tertiary aromatic amines, we refer to the discussion in the present paper. To avoid the use of dry, explosive peroxides, dibenzoyl peroxide moistened with water was replaced with dibenzoyl peroxide damped with 50% dicyclohexyl phthalate, a less volatile compound. We realize that GMA monomer itself is also harmful, has to be handled as a toxic irritant, and may cause allergic skin reactions.

A.R. Spurr: Were any problems encountered in getting the accelerator solution B to infiltrate dense tissues? Since DMBA showed inconsistent embedding results why was it included in your system? Its incompatibility with PEG 200 would also seem to mitigate against its use.
Authors: A disadvantage of N,N-dimethylaniline (DA) is its colour instability. When applied to GMA embedding systems, blocks containing DA resulted in light yellowish blocks that turned into dark brown in time. Bowen and Argentar (1971) reported that 4-tert-N,N-dimethylaniline (DMBA) was the most colour stable aromatic tertiary amine they tested. To take advantage of this feature we tested DMBA. However, we saw that DMBA lead to inconsistent results and thus was less suitable. We did not investigate the depth of penetration of any of the tertiary aromatic amines into tissues.

Reviewer II: The Tg of GMA is said to be 55 °C. The amount of residual cross-linker in GMA will tend to alter that figure. The authors should make clear the purity of GMA used to obtain the 55 °C for Tg.
Authors: Indeed the presence of even small amounts of cross-linker will change the Tg of the ultimate polymer. To avoid unforeseen side effects, we only used highly pure GMA samples. The manufacturer gives the following specifications: GMA > 99%; methacrylic acid content < 0.05%, dimethacrylate content < 0.15%. We did not measure the Tg of pure GMA, but only the Tg's of the final compositions of RES G20.