Oleic Acid-Added Embedding Medium for Histological Analysis of Hard Tissue

JIN IK LIM AND YONG-KEUN LEE*
Department of Dental Biomaterials Science, Dental Research Institute and BK21 Program, School of Dentistry, Seoul National University, Seoul, Republic of Korea

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ABSTRACT For the histological analysis of hard tissue such as bone, various acrylate-based materials have been used as an embedding medium. However, commercial embedding media are expensive, and cutting the embedded block takes a long time. In this study, mixtures of methyl methacrylate (MMA), di-butyl-phthalate (DBP), and oleic acid (OA) were tested for possible application as an embedding medium for large and small undecalcified bone specimens. Mechanical properties were tested in a compressive mode. We investigated the change of hydrophilicity in the sectioned surface by measuring the contact angle depending on the OA. Crystallinity was analyzed using a X-ray diffractometer (XRD). Surface analysis was performed using a confocal laser scanning microscope. To determine the staining efficiency of staining dyes, hamatoxylin-eosin (H&E) and Masson's trichrome (MT) staining methods were performed for the histological analysis of bone-implant complex. We confirmed that the investigated embedding media showed good properties such as optimal mechanical strength appropriate for cutting the embedded block and proper staining efficiency for histological analysis. Therefore, the MMA/DBP/OA mixtures can be used as an embedding media appropriate for various hard tissues and bone-implant complex. *Microsc. Res. Tech. 72:766–771, 2009.*

INTRODUCTION

In recent years, various implant materials, including metals and alloys, have been developed (Bonzani et al., 2006; Geetha et al., 2009; Ma, 2008), and implantation of orthopedic and dental implants has increased (Kao et al., 2007). Osseo-integration is possible between natural bone and several metals and alloys such as stainless steel, vitallium, tantalum, and titanium (Clark et al., 2007; Levine et al., 2006; Lim et al., 2008; Proussaefs et al., 2002; Puleo et al., 2006). These metals and alloys have been widely used as orthopedic and dental implants. To investigate the clinical performance of these materials, suitable evaluation tools should be developed under the same conditions in which the materials are implanted in bone. Because of poor resolution, however, radiographic studies on the bone-implant interface have not yielded conclusive evidence of osseo-integration (Allabouc et al., 1993; Pazzaglia et al., 1994). Instead of radiographic methods, histological methods have been widely used to evaluate the performance of these materials because they are simple and conventional. For the histological analysis, embedded natural tissue, in some cases along with the implant, is necessary. Paraffin is the most commonly used embedding medium for the histological analysis of natural tissue (Conger, 1949; Hine, 1981; Rubin et al., 1983). However, a number of problems with this embedding medium have been revealed. It can be used only for soft tissues after fixation with reagents such as formaldehyde, and it cannot be used for undecalcified hard tissue. However, undecalcified bone section method can provide reliable results for the diagnosis and investigation of bone diseases and regenerated hard tissue (Iwaniec et al., 2008; Schenk 1965; van der Lubbe et al., 1988).

To be used for hard tissues, an embedding medium requires proper hardness and hydrophilicity, compatibility with hard tissue and implant material, and low viscosity and long infiltration time of the embedding medium compatible with high density of bone. To meet these requirements, researchers have performed a variety of studies on hard tissue embedding materials such as methyl methacrylate (MMA) resin, glycol methacrylate (GMA) resin, epoxy resin, or hybrid materials that can substitute for paraffin (Blaauw et al., 1989; Cerri et al., 2003; Fricain et al., 1996; Jonge et al., 2005; Palmieri et al., 2005; Pasyk et al., 1989; Theuns et al., 1993). However, several problems with these media for hard tissue have been observed, such as the high price of the materials and the complicated procedures for slide preparation such as decalcification. In addition to these problems, dimensional changes in embedding medium have occasionally reported in the course of resin embedding step with conventional embedding media. The primary cause of dimensional instability was the transmutation of resins by polymerization at the embedding step, and an accompanying cause was the use of solvent that was

*Correspondence to: Yong-Keun Lee, Department of Dental Biomaterials Science, School of Dentistry, Seoul National University, 28 Yeongeon-dong, Jongro-gu, Seoul 110-749, Republic of Korea. E-mail: ykleedm@snu.ac.kr

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incompatible with resins at the resin-removing step (Salahuddin et al., 2002; Charton et al., 2007).

Mechanical strength and degree of polymerization of embedding resins are directly influenced by plasticizer contents. Di-butyl-phthalate (DBP) is usually used as a nonreactive additive of polymer that acts as a common plasticizer (Rodgers et al., 2005). In cases of excessive amounts of DBP in the embedding resin, several problems have occurred, such as incompletely cured polymer or nonuniform mechanical properties of the inner and outer areas of the resin (Wadey, 2004). Although MMA/DBP mixtures have been used by several researchers (Allen et al., 2006; Parker et al., 2002), the mechanical properties of MMA/DBP mixtures were not optimal for use as an embedding medium for hard tissue because their strength was too high. Therefore, it was reported that new resin compositions should be developed for high efficiency. Commercially, MMA/DBP and plasticized benzoyl peroxide (BPO) mixtures have been used as an embedding medium (Bessho et al., 2001; Ong et al., 2004). However, these commercial embedding media are expensive because both the major component (MMA/DBP) and the plasticized initiator are expensive. Therefore, we added oleic acid (OA) and DBP mixture into the MMA to reduce the strength. OA, an unsaturated fatty acid, has an active double bond that is polymerizable with the double bond of MMA. Therefore, we hypothesized that reduction of strength would be possible by the low crystallinity of poly-(MMA-OA) after the addition of OA into the MMA and DBP mixture. This report describes an optimal embedding medium composed of MMA, DBP and OA.

The purposes of this study were (1) to design an MMA-based material as a novel embedding medium; (2) to determine the influence of composition of the OA and DBP mixture in MMA on the mechanical and chemical properties; and (3) to evaluate the staining efficiency of dye on the embedded bone-implant complex by histological analysis. We studied the compressive strength, hydrophilicity, crystallinity and surface characteristics. In addition, we performed hematoxylin-eosin (H&E) and Massen's trichrome (MT) staining and compared with the results embedded in commercial embedding medium.

MATERIALS AND METHODS

Chemical Reagents

Osteo-bed kit, a commercial embedding medium, was purchased from Polysciences (Warrington, PA, USA). MMA, DBP, OA, BPO, benzoyl peroxide blended with dicyclohexyl phthalate plasticizer (1:1), ethanol, and methanol (99 wt.%) were purchased from Sigma-Aldrich (St. Louis, MO). These chemicals were used without further purification.

Preparation of MMA/DBP/OA Mixtures

As candidates for embedding media, MMA, DBP and OA mixtures of various ratios were fabricated (Table 1) and were polymerized in an oven at 24°C for 3 days after incubation at 55°C for 1 h.

TABLE 1. Concentrations of the MMA/DBP/OA mixture

Composition (wt.%)	MMA	DBP	OA	Initiator (1%)
1	100	0	0	BPO
2	70	30	0	BPO
3	70	29	1	BPO
4	70	25	5	BPO
5	70	24	6	BPO
6	70	23	7	BPO
7	70	22	8	BPO
8	70	20	10	BPO
9	70	18	12	BPO
10	70	15	15	BPO

Measurement of the Amount of Load Under the Same Compressive Displacement

Difference in the amount of load under the same compressive displacement was evaluated according to the OA and DBP contents. The test was carried out using a universal testing machine (Instron model 4467; Canton, MA). Polymerized embedding media, 15 mm in diameter and 10 mm in height, were fabricated. The crosshead speed was set to 1 mm/min, and the load at the point where the specimen was pressed for 3 mm was determined.

Measurement of the Water Contact Angle of the Sectioned Media Block

To evaluate the effect of DBP and OA content on the hydrophilicity of the MMA, the water contact angle of the media block (15 mm in diameter and 10 mm in height) such as polymerized MMA and various mixtures with DBP and OA, sectioned with a band saw cutting system (BS-3000; EXAKT Apparatebau, Norderstedt, Germany) and measured using a contact angle meter (Phoenix 150; Surface Electro Optics, Seoul, Korea). To measure the static contact angle of media, the droplet size should be smaller than the dimension of the specimen. Therefore, deionized water droplets of 20 μl were gently deposited onto the sectioned medium block.

Measurement of the Crystal Intensity of the Media

The crystal intensity diagrams of polymerized MMA/DBP/OA mixtures were measured using a X-ray diffractometer (XRD: D8 advance; Bruker AXS, Karlsruhe, Germany) operating with Cu-Kα radiation ($\lambda = 0.15406$ nm) at 40 kV and 100mA, using a speed of $1^\circ\ \mathrm{min}^{-1}$.

Surface Roughness of the Sectioned Media Block

A confocal laser scanning microscope (model LSM-510 Pascal; Carl Zeiss, Jena, Germany) was used to compare the roughness of the cut surface. The MMA/DBP/OA media, MMA and Osteo-bed were investigated. Roughness measurement and 3D-image analysis were performed using application software (5 Pascal software, Carl Zeiss). The scanning condition was as follows: scan rate: 1 Hz, scan size: 220 × 220 × 30 μm, and scanning speed: 0.8 ms/1 line (512 pixel).

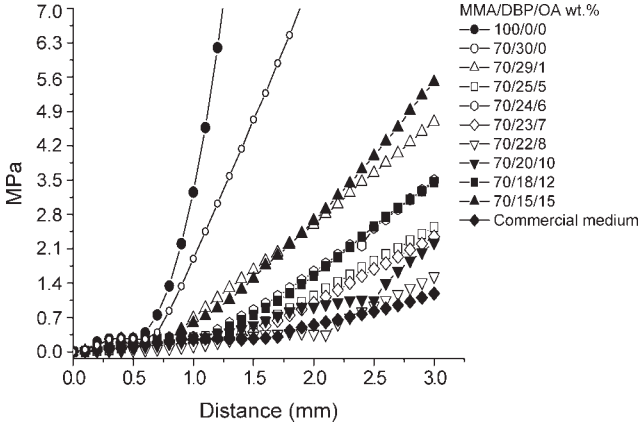

Fig. 1. Differences in the amount of load under the same compressive displacement between the candidate resin mixture and the commercial medium.

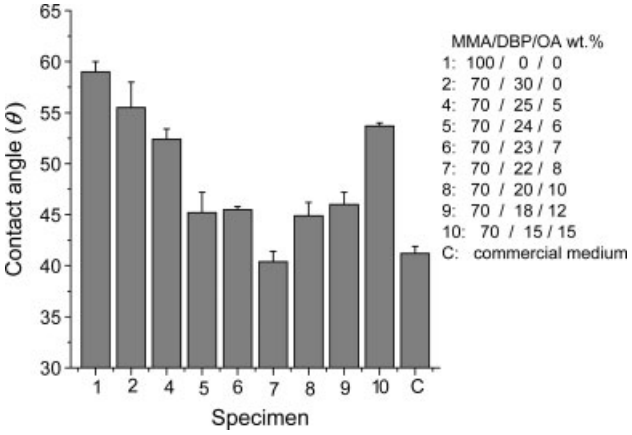

Fig. 2. Differences in contact angle by the DBP and OA addition.

Preparation of Embedded Bone-Implant Complex Block and Histological Examination

Bone-implant complexes were prepared using dental implants. Four weeks after implantation, bone-implant complexes from a dog were fixed in 4% buffered formaldehyde for 24 h and dehydrated in a graded series of ethanol. For embedding, these bone-implant complexes were impregnated in MMA/DBP/OA (80:22:8) mixtures for 1 week at room temperature. For polymerization, the bottles containing the impregnated bone-implant complexes and benzoyl peroxide (1 wt.%) were incubated at 55°C for 1 h and then placed in an oven at 24°C for 3 days. Subsequently, 40 μm sections were cut using a band saw cutting system (BS-3000; EXAKT Apparatebau). At least three sections were made for each evaluation parameter and then stained with H&E and MT. The stained sections were examined using a light microscopy (Nikon Microphoto-FXA; Nikon, Tokyo, Japan) to compare the histological features of the conventional method using the commercial resin and the new method.

RESULTS

The amount of load under the same compressive displacement of MMA/DBP/OA mixtures decreased after the addition of DBP and OA (Fig. 1). MMA or MMA/DBP mixture showed high compressive load when compared with the commercial embedding medium. On the other hand, in case of the DBP 22% and OA 8% mixture, the amount of load under the same compressive displacement was similar to those of the commercial medium. Therefore, we supposed that the optimal medium composition range would be from 20% to 23% of DBP and from 7% to 10% of OA.

The contact angles of MMA and MMA/DBP were 60° and 56°, respectively. However, the contact angles of MMA/DBP/OA mixtures (70/24/6 to 70/18/12) were in the range of 40–45° (Fig. 2). Therefore, MMA/DBP/OA mixtures showed lower contact angles than MMA or MMA/DBP mixtures because of OA. The DBP 22% and OA 8% mixture showed a similar contact angle (40°) to that of the commercial embedding medium.

Crystallization intensity decreased after addition of DBP and OA (Fig. 3). In the small-angle region (10–40°), the diffraction peak around $2\theta = 13°$ was observed in the diffraction profile of the pure PMMA (Wang et al., 2008). Broad band centered around 17° was attributed to amorphous structures of MMA/DBP/OA mixture (Fig. 3). However, the commercial embedding medium showed the lowest intensity in these angles.

The surface roughness of MMA (3.62 ± 0.21 μm) decreased in MMA/DBP/OA mixtures (70/20/10: 1.5 ± 0.037 μm; 70/22/8: 1.14 ± 0.01 μm, Table 2). Although the commercial medium showed the lowest roughness (1.05 ± 0.01 μm), there was no difference with the 70/22/8 mixture.

Photograph of sectioned bone-implant complex embedded in the experimental embedding medium (70/22/8) was compared with those embedded in the commercial medium by low power photograph. Based on microscopic examination, no difference in quality was observed (Fig. 4).

Figure 5 shows a stained photograph of a sectioned slice of bone-implant complex using the 22% DBP and 8% OA resin mixture. It was confirmed that a clear sectioned slice was obtained, demonstrating that effective staining was possible. Additionally clear interface between bone and implant was confirmed and osseointegration of implant by peripheral bone was observed.

DISCUSSION

If an exceedingly hard or soft embedding resin is used, a sectioned slice of high quality would be unobtainable because of noncomplete embedding or low sectioning efficiency caused by the different mechanical and chemical properties between embedded tissue and embedding medium (Iwaniec et al., 2008; van der Lubbe et al., 1988; Schett et al., 2007). Further-

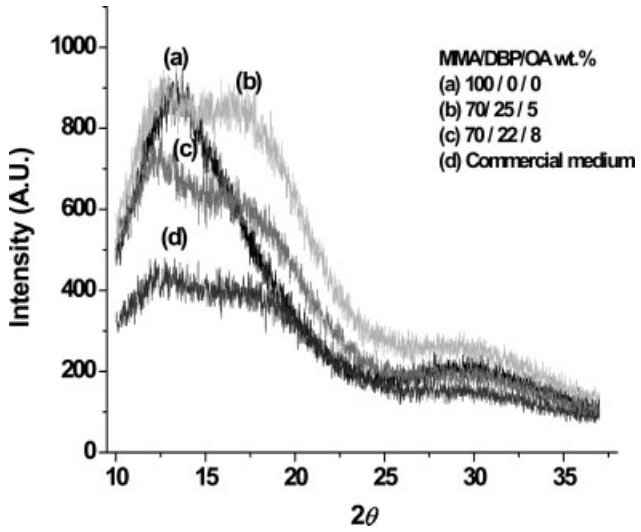

Fig. 3. XRD patterns of the resin mixture.

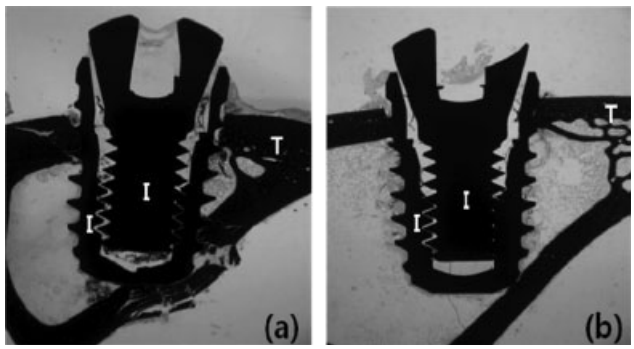

Fig. 4. Sectioned photograph of implanted dog bone using the resin mixture [(**a**) MMA/DBP/OA, 70/22/8; (**b**) Osteo-bed; I: Implant, T: Tissue, ×1].

TABLE 2. Roughness of the sectioned surface using a confocal laser scanning microscope

	MMA/DBP/OA (wt.%)			
	100/0/0	70/20/10	70/22/8	Commercial medium
Roughness, Ra (μm)	3.62 ± 0.213	1.5 ± 0.037	1.14 ± 0.013	1.05 ± 0.012

more, cutting and grinding time and surface roughness of sectioned slice are influenced by the mechanical properties of embedding medium. Therefore, the mechanical properties of embedding media were investigated in this study. Polymer specimens with varied DBP and OA contents were fabricated, and the amount of load under the same compressive displacement was measured through the compressive mode. As the results, mechanical properties of experimental embedding media were similar to those of commercial medium. We supposed that decrease in mechanical properties by OA was caused by low crystallinity.

Most biomaterial and natural tissues show hydrophilic trends (Harnett et al., 2007). Hydrophilicity was measured using a water contact angle meter to determine the compatibility of the embedding medium with the tissue and biomaterial. Before the experiment, we supposed that the new medium would show lower hydrophilicity than the commercial medium because of the hydrophobic property of OA. However, the hydrophilicity of MMA/DBP/OA media increased after the addition of DBP and OA (Fig. 2). We supposed that the hydrophilic functional group of OA was oriented on the outer surface of the resin.

Crystallization intensity ($2\theta = 13^\circ$ in the profile of the pure PMMA) decreased after addition of DBP and OA. We suggest that the broad peak $2\theta = 17^\circ$ was produced by OA and DBP. But peak general intensities in both angles decreased as the OA content increased.

The surface roughness was compared by the DBP and OA contents after the preparation of the specimens under the same condition including band saw and cutting speed. According to the results of surface analysis using a confocal laser scanning microscope with a sectioned slice of thickness 40 μm, Ra (arithmetical average roughness) values of the MMA/DBP/OA media were similar to that of the commercial medium. The high surface roughness of MMA might reflect the scratches made during cutting, which were caused by the high strength of the material.

Regarding the stability of protein in hard tissue after heat treatment at 55°C for the polymerization of MMA/DBP/OA mixtures, protein including collagen was already cross-linked by formaldehyde before embedding step. Therefore, we supposed that denaturation of cross-linked protein by heat treatment should have been ignorable.

We were concerned on the staining efficiency of experimental media because OA was a comparatively hydrophobic material. To evaluate the influence of OA on staining efficiency, histological analysis results based on embedded bone-implant complexes after embedding with the MMA/DBP/OA (70/22/8) mixture and the commercial medium (Osteo-bed) were compared. Dog bone was selected because of optimal size and high strength than those of rat, mice or rabbit. Histological analyses were performed by H&E and MT staining methods. Pink cytoplasm and purple nuclei in tissue were obtainable by H&E staining method. Red keratin and muscle fibers, blue or green collagen and bone, light red or pink cytoplasm, and dark brown to black cell nuclei were obtainable by MT staining (O'Connor et al., 1982; Vahidy et al., 1972; Watts et al., 1981). The commercial embedding medium and the MMA/DBP/OA mixture showed no difference in staining ability. Interface between implant surface and tissue was clearly observed; therefore, developed embedding resin can substitute the commercial resin for histological analysis of hard tissue including implant.

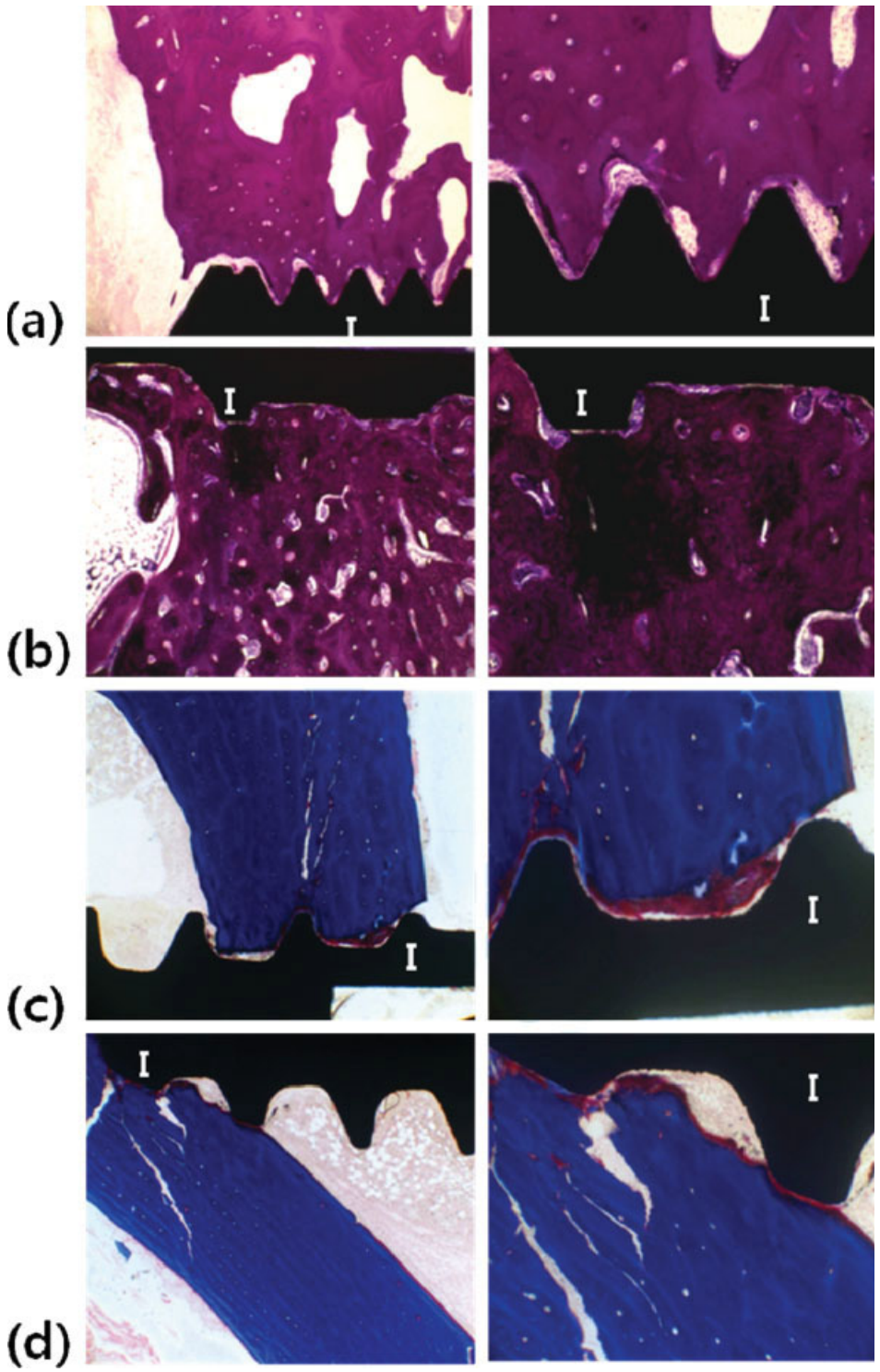

Fig. 5. Histological photographs of dog bone-implant complex embedded in experimental resin mixtures [(**a**, **c**) MMA/DBP/OA, 70/22/8; (**b**, **d**) Osteo-bed; (a, b) H&E staining; (c, d) MT staining; I: Implant, left: ×4, right: ×10].

CONCLUSION

To improve the sectioning efficiency of hard tissue, we studied how to optimize the embedding resin composition for hard tissue using mixtures of MMA/DBP/OA. Surface properties, crystallinity, and chemical and physical properties were determined according to DBP and OA contents, and the efficiency of MMA/DBP/OA mixture was tested by histological analysis. The MMA/

DBP/OA (70/22/8) medium showed adequate mechanical properties and lower surface roughness. The stained efficiency of the new medium did not differ from that of a commercial embedding medium. Therefore, the new medium composition would be widely applicable to various tissue sections including biomaterials. In the future, the new medium composition will be applied to various organs including biomaterials.

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