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Preparation of ground sections using UV-curable acrylic adhesives

S. Dobrovolsky

National Museum of Natural History at the National Academy of Sciences of Ukraine, Kyiv, Ukraine

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National Museum of Natural History at the National Academy of Sciences of Ukraine, Bohdan Khmehnitsky st., 2, Kyiv, 01030, Ukraine. Tel.: +38-044-235-62-66. E-mail: stas000@gmail.com

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Study of ground sections is the most used and, in some respects, still irreplaceable method for examination the microstructure of paleontological and many other hard and friable objects. At the same time, paleontological samples are relatively difficult for preparations of high-quality thin sections. Many techniques and means, particularly embedding media, have been proposed, but they are often hardly accessible, imperfect or insufficiently studied. A promising and easily accessible non-specialized medium, UV-curable acrylic adhesive (glue for glass) was tested for embedding and mounting of objects with diverse mechanical and optical properties. It shows notably good results, in particular durability, reliable adhesion, ease of use and lack of significant birefringence, which makes it especially valuable for polarized light microscopy. Properties of such adhesives are reviewed and compared with properties of epoxy resins and a number of other media. Disadvantages of the adhesives and ways to deal with them are also elucidated. In addition, broadly accessible tools and methods of sawing, embedding, grinding, mounting and other stages of the work are discussed. Efficiency of a number of grinding agents is measured. On the basis of all these results, a technique of making ground sections using easily accessible means was developed and described step by step. The technique was designed for fossil bones, but is applicable to diverse dry samples, including paleontological, neontological and geological ones.

Keywords: ground sections; thin sections; UV acrylics; epoxy resins; embedding media; mounting media; sandpaper; polarized light microscopy.

Introduction

Microstructure of biological and geological objects carries a wealth of information. In particular, histological study of fossil bones is an important source of information about vertebrate animals of past geologic times. Microscopic, as well as macroscopic, characteristics of the bones bear traces of both ontogenesis and phylogenesis (Legendre et al., 2013). Even when bone macrostructure is damaged, microstructure is often well-preserved (Francillon-Vieillot et al., 1989). It can reveal growth history and biomechanical features, sometimes way of life, diseases and other characteristics of the animals (Schultz, 2001; Padian, 2013; Williams et al., 2020).

Despite the invention of non-damaging or less damaging analytic methods, such as X-ray microtomography (Georgiadis et al., 2016), the most available and, in some respects, still irreplaceable (Lovell & Grauer, 2019; Monfroy et al., 2021; Padian & Woodward, 2021) method of microstructure examination is optical microscopy. It deals with thin sections mounted on glass slides. In the case of hard and fragile samples, thin sections are made by grinding and are known as ground sections.

The main steps of ground section preparation have remained constant for two centuries. The first ground sections of modern type were made in the 1810s–1820s by William Nicol, inventor of a well-known polarizing prism used for examination of such sections. Nicol developed his method for the study of fossil wood (Falcon-Lang & Digrius, 2014), but it was quickly applied to bones, teeth (Higham, 1963; Worley, 2009) and rocks, giving birth to petrography (Falcon-Lang & Digrius, 2014). During 200 years, Nicol's technique, while preserving the main steps, was incarnated in countless variations, no one of which is "right": each laboratory works in accordance to its needs, possibilities and findings (Chinsamy & Raath, 1992; Sanderson, 1997; Garcia-Donas et al., 2017; Heck et al., 2020).

Fossil bone is a relatively difficult object for preparation of highquality thin sections. It differs from recent ones by brittleness, lower birefringence, often by higher density and attached rock, as well as by high value. All these features complicate the work. But methods developed for fossil bones are suitable for the majority of other biogenic and abiogenic hard objects: dry recent bone, eggshell, teeth, fossil wood, most rocks etc.

Means for preparation of ground sections, primarily embedding media, are quite diverse and often hardly accessible, imperfect or little studied. This encourages further research in the area. A number of works have shown the potential of non-specialized embedding media: acrylic adhesives with ultraviolet curing (Noetinger et al., 2017; Strüssmann et al., 2018; Phillips, 2020). Some of them are designed specifically for bonding glass. Preliminary tests by the author have shown them to be the only widespread substances which are well suited for preparation of durable ground sections suitable for both usual and polarized light microscopy. Taking this into account, the described technique was established, aimed at production of high-quality sections of fossil bones using widely accessible means.

Material and methods

The described method was developed mostly on the bones of recent chicken and successfully tested on bird bone fragments from the Eocene location Ikove (Ukraine, Luhansk region) from the collection of National Museum of Natural History at the National Academy of Sciences of Ukraine. For broader view, samples with dissimilar optical and mechanical properties were also tested, including recent eggshell, nutshell, quartz sand and single quartz crystal.

A number of easily available liquids were tested for embedding. Their suitability was evaluated in view of the needs of usual and polarized light microscopy and the prospects for long-term stability of the result. Among the acrylic UV adhesives for glass, the brand Loxeal 30-23 (produced by Loxeal, Italy) was chosen, because it can be considered as a compromise in view of the requirements of low viscosity in liquid state and high hardness in solid state. Additionally, the most widespread in Ukraine UV adhesive for glass was tested: Kafuter, also known as "Fixator" and "Kemil" (produced by Kafuter Co. LTD, China). For comparison, 3 other easily accessible media were examined: epoxy resin Magic Crystal 3D

(supplied by Dekopark, Ukraine), notable for relatively low viscosity and long time before hardening, cyanoacrylate adhesive "Sekunda" and acetone solution of poly(methyl methacrylate), also known as acrylic glass.

The micrographs were taken with Sony NEX-5 camera attached to Biolam S-11 microscope without microscope eyepiece and camera lens. Polarized light microscopy was implemented using a homemade device made of linear polarizers for mobile phones. Unlike usual polarizing microscopes, it is designed for rotation of the system "polarizer + analyzer" instead of the slide, because immobility of the slide enables easy comparison and combining of the images. In addition, such construction does not require centering and strain-free objective (because the analyzer stands before the objective in the path of light).

Image processing, including flat-field correction and focus stacking, was done by self-made scripts, except for decoding raw files, which was done by Dcraw program (www.dechifro.org/dcraw) and demosaicing which was done by scripts from LASIP Project (http://webpages.tuni.fi/lasip/cfai). The matrix of colour conversion to the standard sRGB space was calculated according to the algorithm from Adobe DNG format specification (version 1.4.0.0).

Making of a thin section must be preceded by documentation of the object: photographing, measurement and, if appropriate, making of casts (Lamm, 2007, 2013) or digital 3D models. Location of the planned sample in the object must also be recorded. Then the sample is cut off from the object, embedded in some liquid, which subsequently solidifies, ground on one side, glued to microscope slide, microphotographed if appropriate, ground from the other side and covered by coverslip. These steps are described in the following sections.

Preparation of thin sections using the described technique requires:

– for embedding and curing: the above-mentioned adhesive, plastic syringe with diameter 1–2 cm larger than the sample, transparent vacuum chamber and vacuum pump, plastic container for the adhesive with sufficient volume to fill the sample, boiling chips, plasticine, UV lamp, sheet of black paper;

 for grinding: sheets of sandpaper with grit sizes P400–P2500, vacuum cleaner, metal mesh with cells several millimeters wide, a sheet of thick glass with the area of about ten square decimeters, water sprayer;

 for gluing to the glass and covering with cover glass: slides and coverslips, distilled water, lighter, a sinker of several grams, a needle, a bright lamp, preferably a polarizing microscope and an air purifier;

- rubber (non-slippery) gloves, respirator and goggles with UV protection.

Sectioning of the sample

Paleontological and geological samples are usually brittle, so their cutting requires especial caution. To prevent destruction, it is preferable to cut samples after embedding, but it is not always possible. The planned place of sawing can be covered with some hard coating that will not hinder microscopy (e.g., a varnish which can be dissolved later). For minimizing material loss (kerf loss), the saw should be as thin as possible.

Hard samples can be sliced in several ways. The most accurate, although uncommon one, is laser microtomy, which gives kerf width in the order of hundredths of a millimeter. It is reported to be applicable to translucent materials, including bones and teeth, at least recent ones (Lubatschowski, 2007; Fornaroli et al., 2015; Boyde, 2018). Much more common is using of disk saws, or, in the case of large objects, core drills (Stein & Sander, 2009), usually with powdered diamonds. Thicknesses of the disks start from 0.02 mm (Franssila, 2010; Zhou et al., 2012) with sporadic reports of values 0.010-0.015 mm (Arnold, 1958; UKAM Industrial Superhard Tools, www.ukam.com), but such disks are very fragile and suitable only for thin objects (being used mainly for cutting of semiconductors in the electronics industry). In paleontology and neontology, disk or band saws (or coring bits) tenths of a millimeter thick are used (e.g., Donath & Rohrer, 2003; Stein & Sander, 2009; Lamm, 2013; Wang et al., 2013; Haas & Storå, 2015). Less common instruments are wire saws, whose thickness varies in the same boundaries: from 0.02 mm (rarely) to several tenths of a millimeter (Wilson, 1994; Haridy et al., 2021). Disks 0.2-0.3 mm thick are described as ultra-thin (Goodwin & Horner, 2004; Yuan et al., 2018). At the same time, small samples can be sectioned by a folk sawing method using a double-edged safety razor blade serrated by strokes of a knife. Thickness of such blades is 0.1 mm and can be easily reduced even more via grinding between two glasses, but it threatens deformation during sawing.

The result can be significantly improved by stringing the blade in a fretsaw-like frame and constraining its movements to single plane with the help of guiding plaques. Adding of a device for moving the sample perpendicularly to this plane (Fig. 1) enables one to obtain a sequence of parallel slices with controlled thickness (Fig. 2).

Samples which are not too brittle (like recent bones) can be sliced with interval of several tenths of a millimeter. It enables reconstruction of 3D structure with the same depth resolution (e.g., Fiala, 2005).

Some authors moisten samples with water, oil or glycerol before cutting and grinding, but it facilitates fracturing of brittle samples. In addition, the liquid absorbed by the sample may create difficulties later (Miller, 1988; Horner & Lamm, 2011). Sawing of hard objects is greatly facilitated by adding of abrasive powder, which must be fine enough. Ash from burnt worn sandpaper P1000 and finer ones is suitable. However, it also increases wear of the saw.

The surface of the sample should be flat to avoid the need for deep grinding after embedding, because the best impregnation by embedding liquids is achieved in outer layers (tenths of a millimeter in dense samples), and these layers should not be erased. The surface of the ready slice can be flattened with a sandpaper placed on a flat hard surface. Non-embedded brittle samples, including fossil bones, must be ground extremely carefully and with paper not coarser than P1000.



Fig. 1. Double-edged safety razor blade, serrated by strokes of a knife, and a device for sawing with such blade (thickness of the slices can be controlled by movement of the object stage with the help of the wheel in lower left)



Fig. 2. Tibiotarsal bone of a recent chicken sliced in the described way

Cleaning the sample

Paleontological and geological samples do not require fixation or other special preparation for embedding, but must be cleaned from contaminations and water (Cho, 2012). Water is insoluble in main embedding media (majority of acrylic and epoxy resins, Canada balsam etc.) and creates cavities or fog (Ries, 2003; for the exceptions see Glauert & Lewis, 1999; Schultz, 2012; Hand, 2013). Sand should also be removed, because it litters the image in polarized light due to strong birefringence.

Fatty contaminations can be removed by various volatile non-polar or weakly polar solvents (white spirit, ethanol etc.). It is important to note that acetone dissolves many adhesives and can cause break-up of restored objects. Some authors also report its harmful influence on fossil bone itself (Heck et al., 2020). Before using the solvent, it is desirable to check it for absence of non-volatile fraction by evaporating a drop on a glass. For more complete cleaning, different liquids can be used consecutively. Centimeter-sized fragments of fossil bone can be held in a solvent for one or several days with occasional replacement of the solvent and moderate heating (e.g., on a central heating battery).

After washing, the solvent must be removed (remnants of ethanol and acetone can hinder curing of embedding media (Mollenhauer, 1993; Glauert & Lewis, 1999). Evaporation of the solvent would be faster and more complete in the case of multiple vacuuming. This would also remove water to some extent. Desiccation of the samples can be improved by heating in a hermetic container with anhydrous calcium chloride.

Main properties of embedding media

For preparing a thin section, a hard sample must be embedded in some liquid that subsequently hardens and supports it during grinding (and, possibly, sectioning).

Recent bones do not always require complete impregnation: if osteocyte lacunae and canaliculi remain air-filled, they are more prominent, and sometimes this is pursued consciously (Enlow, 1954). In fossil bones, difference of impregnation level, which is sometimes seen between neighbouring osteons and even lamellae of one osteon, can accentuate these features quite well (Figs. 3, 4). In particular, borders of secondary osteons are often underimpregnated, probably, due to strong mineralization (Skedros et al., 2005).



Fig. 3. Thin section of fossil bone (cf. Dasornis, Aves: Pelagomithidae, humerus, Eocene of Ikove, Ukraine) embedded into UV-curable adhesive for glass; left: view in usual light; incomplete impregnation (dark regions) reveal details of osteon structure which are not always revealed even by polarized light (at the right; crossed linear polarizers with waveplate); width of each image is 1.4 mm, the section is ~60 µm thick



Fig. 4. Thin section of fossil bone fragment (Aves: Pelagornithidae indet., tibiotarsus, Eocene of Ikove, Ukraine) incompletely impregnated with UV-curable adhesive for glass; left: view in usual light; underimpregnation elucidates layers of osteons which aren't revealed in other ways, including polarizing microscopy (at the right; equivalent of view between circular polarizers); width of each image is 1.8 mm, the section is ~50 µm thick

However, in most cases impregnation must be close to complete, so, the embedding medium must be fluid enough. Low viscosity is important also because it lets air bubbles easily pop up and burst.

When solidified, the embedding medium must become approximately as hard as the sample but not fragile, and should have as little shrinkage as possible. In addition, it must be able to be securely glued to a glass slide and coverslip.

Not all embedding/mounting media show good adhesion to glass. This is further complicated by air moisture (water gradually diffuses through any polymer and weakens the adhesion) and by stresses arising from shrinkage of the polymer due to curing and changes in its volume caused by changes of temperature and humidity (Chang et al., 1997; Davison, 2003; Neuhaus et al., 2017). The coefficient of thermal expansion of most polymers, including acrylic and epoxy resins, lies in the range $(50-100) \cdot 10^{-6} \text{ K}^{-1}$, which is an order of magnitude higher than that of glass (The Engineering ToolBox, engineeringtoolbox.com). Adhesion can be improved by pre-treating the glass with silanes (Caropreso et al., 2000; Davison, 2003) or by other sophisticated methods and, to some extent, by frosting. Often gluing is performed by special substances other than the embedding medium (Lamm, 2013; Garcia-Donas et al., 2017), but it raises questions about reliability of these substances (Cho, 2012; Neuhaus et al., 2017) and can increase mechanical and optical heterogeneity of the slide. Alternatively, plastic slides can be used instead of glass ones (Bauer & Mahovlic, 2003; Lamm, 2013; Garcia-Donas et al., 2017), but they are less stable and long-lasting (Neuhaus et al., 2017) and can be birefringent (Boyde & Bromage, 2021).

Among optical properties of embedding media, the most important ones are refractive index and optical activity. The closer the refractive indices of the object and medium, the more transparent is the object and the more prominent are its regions which differ by absorption coefficient. Equality of the refractive indices can even render macroscopic pieces of recent bone transparent, but under the difference of 0.05-0.10 transparency ceases (Berke et al., 2016). During microscopic observation, a difference of 0.05 gives the object moderate contrast and 0.10 - high contrast (Neuhaus et al., 2017). Even difference of several hundredths in refractive index can significantly affect visibility of embedded objects (Phillips, 2020). Many objects require embedding medium with the same refractive index, but in the case of fossil bones it does not seem to be desirable and can make under-impregnated places and other flaws the most prominent features.

Refractive index of recent bones for visible light constitutes 1.55–1.56 (Rogers, 1924; Ascenzi, 1949; Ascenzi & Fabry, 1959), and that of their main mineral constituent, hydroxyapatite, 1.64–1.66 (Anthony et al., Handbook of Mineralogy, Mineralogical Society of America). Refractive index of fossil bones depends on the chemical environment during fossilization and usually amounts to 1.57–1.62, most frequently 1.60–1.61 (Rogers, 1924).

If the refractive index of the embedding medium matches that of the cover glass, the adhesive for its gluing and (if used) immersion oil, this improves image quality (James, 1976; Noetinger et al., 2017). The standard refractive index of coverslips is 1.5255 ± 0.0015 (ISO 8255-1:2017), slides -1.53 ± 0.02 (ISO 8037-1:1986), and immersion oil -1.5180 ± 0.0005 (ISO 8036:2015; all standards relate to the wavelength of 546.07 nm).

The refractive index of the embedding medium can be measured with the help of a microscope. It requires a plane-parallel sample of known thickness and measurement of the objective displacement needed to shift focus from one surface of the sample to another (Duc de Chaulnes method):

$n_s = n_a \cdot d/\Delta h$,

where n_s is refractive index of the sample, n_a is refractive index of air (1.0003), d is thickness of the sample and Δh is vertical travel of the objective which can be calculated from readings of the fine adjustment knob (Garbovskiy & Glushchenko, 2017).

The equation is correct in the paraxial approximation (for the rays which make small angles to the optical axis), so it works most accurately with weak objectives. Its generalized form, applicable to any objectives, also exists (Miller, 1968; Visser et al., 1992), but the best results are obtained from calibration of the method using substances with known refractive indices. This method can give error about 0.01 or, with calibration, even smaller (Lawless & DeVries, 1964; Deshpande et al., 1980).

The same formula allows one to determine thickness of the samples with known refractive index. This approach can give better precision than mechanical micrometers (Bromage & Werning, 2013) and in the case of ready samples (embedded between glasses) becomes irreplaceable.

Since examination of bones and many other hard objects is greatly advanced by the use of polarized light microscopy (Haas & Storå, 2015; Georgiadis et al., 2016), cured embedding medium should lack optical activity (ability to rotate the plane of light polarization). This allows one to see the sample's optical activity in pure form. It is especially important for fossil bones since their optical activity (specifically, birefringence) is heavily reduced due to loss of collagen which is responsible for most of the bone birefringence (Tonna, 1964). Embedding media can become birefringent because of deformation (photoelasticity effect), in particular due to shrinkage which accompanies solidification.

Embedding media used in light microscopy

Historically, the first embedding and mounting medium for any hard samples was Canada balsam (Witham, 1831; Falcon-Lang & Digrius, 2014). It proved its reliability (some mounts already persist for almost 200 years) and is sometimes used until now despite a number of drawbacks, e.g. need for toxic solvents, incompatibility with remnants of water, gradual yellowing and high price. These drawbacks were mostly

overcome by the invention of its analogue Euparal (Brown, 1997; Ravikumar et al., 2014; Neuhaus et al., 2017). Another long-used embedding medium for solid samples is celloidin (Plowman, 1904), but it is hardly suitable for bones (Arnold, 1951; Woodruff & Norris, 1955; Callis, 2002). In 1937, use of poly(methyl methacrylate), also known as acrylic glass or plexiglass, was proposed (Hibben, 1937). It is notable for good infiltration, high hardness and later became one of the most widespread mounting media for bones and other hard samples. In 1960, Rosenberg et al. proposed an analogous medium: poly(glycol methacrylate), which is inferior in hardness and permeability, but does not require deep dehydration of the sample, has 3 times lower shrinkage and turned out to be also well suited for recent and fossil bones (Cole & Sykes, 1974; Wong, 1985; Garland, 1989; Gerrits & Horobin, 1996; Erben, 1997; Hand, 2013). These and other acrylic compounds became the basis of a number of embedding media for electron and light microscopy (Glauert & Lewis, 1999; Stirling & Woods 2019).

In the middle of the XX century, several other media for ground sections became widespread. For instance, waxes-based thermoplastic medium Lakeside 70 came into use in petrography, but its use is somewhat limited due to the need of heating to ~130 °C (Dalrymple, 1957; Wells, 1989; Lamm, 2013). Researchers in other fields, primarily electron microscopy, developed a number of epoxy resins which came into use in light microscopy as well: Araldite, Biodur, Epon, EpoThin, Spurr's resin etc. (Glauert & Lewis, 1999; Schultz, 2001; Neuhaus et al., 2017; Stirling & Woods, 2019). This class of media has low shrinkage, high durability and a number of other advantages (Nye et al., 1972; Pohl & Browne, 1973; Mollenhauer, 1993; Marks et al., 1996; Glauert & Lewis, 1999; Schultz, 2012). Electron microscopy provided also polyester resins, which have approximately the same efficiency as epoxy resins, but much lower cost (Heck et al., 2020). These resins, introduced in the 1950s, lost their popularity for some time, but later became the most widespread media for fossil bones (Litwin, 1985; Hand, 2013; Lamm, 2013; Heck et al., 2020).

Some acrylic and epoxy resins can be cured by ultraviolet radiation (Sanderson, 1995). In the late 1970s, acrylic and urethane-acrylic UVcurable adhesives for household and industrial use, including glass bonding, came into use and later became widespread (Bachmann & Cantor, 1999). In 1986, such non-specialized media were shown to be suitable for making thin sections of soft tissues (Silverman, 1986) and rocks (Yanguas & Paxton, 1986). The following year, their suitability for mounting of recent non-decalcified bone sections, as well as for gluing cover glass, was reported (Denton, 1987; Garland, 1989; Neuhaus et al., 2017). In the late 1990s, UV adhesives were already used for embedding various objects by some microscopy amateurs (Brinkworth & Smith, 1997. Mounting made easy! Micscape, www.microscopy-uk.org.uk/mag/ art97/locmount.html). A number of recent studies have shown good suitability of such media for pollen samples (Noetinger et al., 2017), wool, calcareous and siliceous microfossils (Phillips, 2020), fish otoliths (Strüssmann et al., 2018) etc. Introduction of these adhesives to industrial and household practice was enhanced by their relative safety along with fast and controlled curing (Bachmann & Cantor, 1999; Goss, 2002). Their additional microscopy-related advantages are absence of solvents and hardeners, low viscosity, no need for heating, satisfactory chemical inertness in cured state, including neutral pH and compatibility with dyes, high hardness and transparency, intentionally strong adhesion to glass (e.g., due to silane additives: Goss, 2002; Vitale et al., 2018), refractive index close to that of glass (Tennent & Townsend, 1984; Punge, 2009), absence of fluorescence and absence of significant deterioration with time (including crystallization, leakage and damaging by living organisms), traced during 35 years in some cases (Silverman, 1986; Neuhaus et al., 2017; Noetinger et al., 2017; Strüssmann et al., 2018; Phillips, 2020).

Efficiency of many embedding/mounting media is explored insufficiently, and they are often chosen not on the basis of properties, but on the basis of familiarity and price: specialized media are often expensive (Heck et al., 2020). No medium is completely satisfactory (Mollenhauer, 1993; Ellis, 2003b; Ravikumar et al., 2014). Wrong choice of the medium caused loss of many scientifically important samples (Brown, 1997; Neuhaus et al., 2017). So, comparative tests of various embedding media, especially widely accessible ones and especially in respect of longevity, retain importance (Neuhaus et al., 2017; Heck et al., 2020).

Properties of UV-curable acrylic adhesives

Loxeal 30-23 is a representative of UV-curable acrylic adhesives. According to the manufacturer, it contains 2-hydroxyethyl methacrylate (glycol methacrylate), isobornyl acrylate, acrylic acid and diphenyl (2,4,6trimetylbenzoyl) phosphine oxide. The first three compounds are widely used as monomers in the synthesis of polymeric materials, the latter is a photoinitiator. Glycol methacrylate constitutes the basis of some embedding media for electron and light microscopy (Cole & Sykes, 1974; Garland, 1989; Gerrits & Suurmeijer, 1991; Gerrits & Horobin, 1996). Isobornyl acrylate is used as a reactive diluent; it has low viscosity, but its polymer shows high hardness, chemical resistance and glass transition temperature (Zeggai et al., 2018; Lastovickova et al., 2021; Ossowicz-Rupniewska et al., 2021). Viscosity of acrylic acid is even lower. The ratio of adhesive components is not reported. However, manufacturers of specialized embedding media do not disclose the exact composition as well (Singhrao et al., 2011; Neuhaus et al., 2017).

Acrylic media are notable for their relative health safety, although they are somewhat irritating and allergenic (Punge, 2009; Tobler & Freiburghaus, 1990). This distinguishes them from many others media that are toxic themselves, like epoxy resins, or need toxic solvents, like Canada balsam.

According to the manufacturer, the adhesive can be stored in unopened packaging at 25 °C for one year. According to the author's observations, it practically does not change its properties even 1.5 years after opening (when stored at ~20 °C). The adhesive is fully suitable for vacuumizing, even long-lasting (at least several hours under ~0.02 atmospheric pressure). Keeping it in the light should be avoided: in several hours even dim indoor light causes its thickening, although during 10–20 minutes even bright white LED lamp is harmless.

Viscosity of Loxeal 30-23 at 25 °C is 0.05–0.10 Pa·s (Technical Data Sheet for Loxeal UV 30-23), which is a moderately low value for embedding media (Lamm, 2013). It roughly equals the viscosity of plant oils. Low viscosity is a common feature of acrylics (Glauert & Lewis, 1999; Hand, 2013; Stirling & Woods, 2019). It enhances impregnation of the samples and reduces the problem of air bubbles: in this adhesive they quickly pop up and burst in a few minutes.

Polar acrylic media (including glycol methacrylate-based) differ from other acrylics, most epoxies and many other embedding media in the absence of the need for deep dehydration of the sample, because they are able to dissolve some water (Glauert & Lewis, 1999; Punge, 2009). Water content up to a few percent even does not reduce hardness of poly(glycol methacrylate) significantly (Rosenberg et al., 1960). Liquid Loxeal 30-23 adhesive is also mixable with ethanol and remains workable after evaporation of the latter, which is of interest for embedding soft biological samples by the way of replacing water with the adhesive through intermediation of ethanol.

When cured, the UV adhesive, like other similar media (Macret & Hild, 1982; Singhrao et al., 2011; Ravikumar et al., 2014), apparently forms a thermosetting cross-linked polymer, because it does not melt when heated or dissolve in solvents, but only somewhat softens and swells in polar liquids, including water and ethanol. Their absorption is a known property of the polymers of glycol methacrylate and acrylic acid, which are part of the adhesive (Cole & Sykes, 1974). Its insolubility makes it unsuitable for the cases when embedding must be reversible. But sections made on the UV adhesive do not require storing in horizontal position, because, unlike thermoplastic media such as Canada balsam (Neuhaus et al., 2017), it cannot flow.

Hardening of acrylic UV adhesives is inhibited by atmospheric oxygen (due to binding of free radicals involved in the polymerization chain reaction). This makes the surface layer (~0.1 mm thick) sticky. Acetone affects the reaction in the same way (Glauert & Lewis, 1999; Hand, 2013). To some extent, this can be prevented by using high-intensity short-wave UV radiation (Goss, 2002) or by curing in an oxygen-free environment. During curing, acrylic media significantly shrink and heat up (Glauert & Lewis, 1999). This can cause growth of gas bubbles and requires gradual curing from the bottom upwards. Loxeal 30-23 adhesive shrinks by 9% (author's measurements). This is slightly higher than shrinkage of pure glycol methacrylate (~6% according to

Rosenberg et al., 1960), but much less than the shrinkage of methyl methacrylate (21% according to Patel et al., 1987) and various media which harden due to solvent evaporation. Polymerization and shrinkage partly occur before solidification, and this part of the shrinkage is not harmful. Increasing it (known as prepolymerization) can reduce further (undesirable) shrinkage. For example, prepolymerization of liquid glycol methacrylate can reduce shrinkage during curing to 1% (Cole & Sykes, 1974; Wong 1985). Prepolymerization of the UV adhesive can be reached by keeping it under usual room lighting during several hours. It makes the adhesive viscous and should not be performed before impregnation of the sample. However, curing from the bottom up, as described below, makes prepolymerization unnecessary.

According to the manufacturer, Loxeal 30-23 reaches its final hardness in one day. The author's measurements by the Brinell method (indentation of a steel ball) show that the hardness practically reaches final level in the first hours after curing, but microscopic shrinkage, judging from slight bending of polished surfaces, lasts for days and weeks. This is probably caused by continued polymerization (similar behaviour of glycol methacrylate and UV adhesives has been reported in the literature: Casey et al., 1988; Clements, 2006; Hand, 2013). The higher the curing temperature, the greater part of polymerization occurs immediately (Hand, 2013).

Hardness of the cured adhesive is 65–75 units of Shore D scale. This is roughly equal to the values for firm plastics. Despite rather high hardness, the adhesive is not brittle: under stretching testing, deformation before rupture reaches 60–100%. The cured adhesive withstands temperatures from -55 to +120 °C (Technical Data Sheet for Loxeal UV 30-23).

Mechanical properties of the adhesive have proved to be suitable for making ground sections of fossil bone (Fig. 5, 6). Objects with sharply different mechanical properties did not reveal any problems either: neither eggshell, which is more fragile and requires at least two times thinner sections (Fig. 7), nor quartz sand, which is much harder and the section of which was prepared almost three times thicker (Fig. 8). In addition, a section of 1×2 cm sized quartz crystal was prepared in the same way and successfully used as a full-wave plate for polarized light microscopy of fossil bones (Figs. 3, 6). This requires sectioning along axis of the crystal and grinding to a thickness of 60–65 µm. In all cases, support provided by the adhesive was strong enough to prevent disruption of the sample during grinding.

Binding of the adhesive to glass does not cause any complaints (after nearly three years, no exfoliation, peeling or other deterioration of sections glued to both frosted and polished glass was observed).

The refractive index of the cured adhesive, measured with the Duc de Chaulnes method (45 days after curing; vacuumed before curing), is 1.51 ± 0.02 (according to the manufacturer, 1.48-1.51). This virtually matches standard values for microscope slides (1.53 ± 0.02) and coverslips (1.5255 ± 0.0015), as well as for immersion oil (1.5180 ± 0.0005 for the wavelength 546.07 nm). Such matching of refractive indices enhances image quality. Refractive indices of other acrylic media are very close: 7 brands studied by Punge, 2009 showed values from 1.49 to 1.51, and 14 samples studied by Tennent & Townsend, 1984 – from 1.466 to 1.505.

Birefringence of cured UV adhesive is negligible and does not hamper observation of sections under polarizing microscope. Only a weak birefringence at the edges of hard objects is sometimes present (Fig. 5, 6).

A drawback of the tested adhesive is the ability to form microscopic needle-like crystals, almost invisible in usual light, but brightly shining under a polarizing microscope. They appear in thin layers of liquid adhesive (especially on the edges of wetted regions) after several minutes in the open air or vacuum. Apparently, these crystals consist of some nonvolatile constituent which crystallizes due to evaporation of volatile ones. Quick work prevents formation of the crystals and they rarely become a problem. While the adhesive is liquid, they can be dissolved by heating.

The widely accessible adhesive Kafuter shows similar properties to Loxeal 30-23 and is also suitable for thin section preparation. However, it differs by noticeably higher viscosity and shows some muddiness at the time of curing, although this quickly disappears.

For broader view, the UV adhesive was tested, in addition to fossil and recent bone, on samples with highly different mechanical and optical properties: quartz and recent eggshell. Eggshell is an example of object consisting of calcium carbonate (in the form of calcite). Since biology, including paleontology, deals with plenty of such objects, question of suitability for carbonate samples is important for any method of thin sections preparation. By comparison with calcium hydroxyphosphate, the main mineral of bones, calcium carbonate is more fragile and birefringent; in addition, eggshell has lower porosity and translucence. All these differences complicate the work: the mentioned optical properties require thinner sections, and the mechanical ones require gentler grinding. Carbonate objects acquire scratches more easily, which, in addition, are more prominent on thinner sections.

The tested embedding medium turned out to be suitable for the eggshell (Fig. 7). The sections show details of its structure, and the polarizing microscope reveals individual crystals of calcite which differ by apparent brightness and colour. Other things being equal, the colour is determined by tilt of optic axis of the crystal to the line of sight, and the brightness depends also on its orientation relative to transmission planes of polarizer and analyzer. The crystals are seen to extend over nearly all thickness of the eggshell and have their axes oriented nearly randomly.

Quartz (Fig. 8) is an example of extremely hard and strong material. The used embedding medium turned out to be hard enough to provide it with sufficient support during grinding. In addition, this medium does not acquire noticeable birefringence even on the edges of hard objects and even in a rather thick section (does not demonstrate photoelasticity caused by shrinkage-related deformation). Consequently, it allows one to observe optical activity of embedded sample in pure form.

Properties of epoxy resins

Epoxy resin Magic Crystal 3D, taken for comparison, turned out to be less suitable for microscopy for several reasons. Most of these disadvantages are common features of epoxy resins. Some advantages also exist.

The suppliers recommend using the resin in 1.0–1.5 months (Elastoform, elastoform.com.ua; DecoPark, decopark.com.ua). The author did not notice significant change in its properties even after 3 years, but there is evidence that expired resins may poorly polymerize, which is not immediately apparent (Davidson & Alderson, 2009). Typical shelf life of epoxy resins is 0.5–1.0 year (Clements, 2006; Davidson & Alderson, 2009).

For use, epoxy resin is mixed with a hardener. After mixing, it remains liquid for some time (40–60 minutes for the examined brand according to the manufacturer, and even longer according to the author's observations). In epoxy resins in general, this time varies at least from minutes to days (Clements, 2006). UV adhesives for glass can remain liquid indefinitely, which is beneficial for impregnation.

Epoxy resins are usually quite viscous, which additionally complicates impregnation (Mollenhauer, 1993; Stirling & Woods, 2019) and causes the problem of air bubbles, which easily appear during mixing, slowly pop up and almost do not burst. However, the viscosity can be significantly decreased by heating, and rising of the bubbles can be facilitated by vacuuming. The viscosity of epoxy resins common in microscopy (at 25 °C) varies in the range of 0.065-3 Pa s (Stirling & Woods, 2019), and typical general-purpose brands have viscosity on the order of 10 Pas (Selwitz, 1992; Unnikrishnan & Thachil, 2006). The lowest value for epoxy resins is in the order of 0.01 Pa s, but such resins are less stable and more toxic (Selwitz, 1992; Glauert & Lewis, 1999; Horie, 2010; Stirling & Woods, 2019). The resin Magic Crystal 3D has a viscosity of 2.5 Pa s at 25 °C, and its hardener (which must be mixed in the proportion of resin:hardener = 10:6 by weight) has 0.15 Pa s (supplier data), while the value for Loxeal 30-23 adhesive is only 0.05-0.10 Pa s (Technical Data Sheet for Loxeal UV 30-23).



Fig. 5. Thin section of a fossil bone embedded in the adhesive Loxeal 30-23: a – view in plane-polarized light (without analyzer), equivalent to usual light; b – between crossed linear polarizers; c – equivalent to view between circular polarizers (pixel-wise maximum of brightness from 4 images taken with different orientations of crossed polarizers); transmission directions of the polarizer and analyzer are shown with thick and thin lines in the circle respectively; transverse section of humerus of a bony-toothed bird (Pelagomithidae, cf. *Dasornis*) from Eocene of Ikove, Luhansk Oblast, Ukraine; inner side of the bone wall is at the left; size of each image is $3.0 \times 1.4 \mu m$; the section is approximately 60 μm thick; mounted on a slide 1.9 mm thick, rubbed with fine sandpaper for better adhesion



Fig. 6. Transverse section of femur of a bony-toothed bird (Pelagomithidae, cf. *Lutetodontopteryx tethyensis*) from the same locality, processed in similar way: *a* – view in plane-polarized light (without analyzer), equivalent to usual light; *b* – between crossed linear polarizers with full-wave plate (made of quartz crystal processed in similar way); white lines in the circle show directions of transmission of the polarizer (thick) and analyzer (thin); red and blue lines show directions of bone fibers highlighted in these colours by the waveplate



Fig. 7. Thin sections of recent hen eggshell, embedded in the adhesive Loxeal 30-23: both sections are made from the same brown egg; left: in plane-polarized light (without analyzer); right: between crossed linear polarizers (directions of transmission are shown); the scale is identical everywhere; above: a section in the plane tangential to inner surface of the eggshell (inner layers are sectioned in the lower left and outer layers in the upper right); a sample from the egg's equator, sharp end of the egg is rightwards; the section is 20–25 μm thick and mounted on a slide 1.8 mm thick, rubbed with sandpaper P2000 for better adhesion; below: a section perpendicular to surface (along a meridian) near the equator; the sharp end of the egg is to the lower right; thickness of the section is approximately 30 μm, mounted on a slide 1.8 μm thick, frosted for better adhesion



Fig. 8. Thin section of quartz sand (Kyiv, bank of Dnieper river at Slavutych metro station, collected 6 May 2020), embedded in the adhesive Loxeal 30-23: a – in plane-polarized light (without analyzer); b – between crossed linear polarizers; c – equivalent of view between circular polarizers (see Fig. 5); video of the sample under different illumination is available at www.youtube.com/watch?v=fgsYYqvR7mc. Size of each image is 3.26×1.47 mm; the section was made approximately 160 µm thick for the best manifestation of interference colours (other things being equal, the colours reflect tilt of optic axis of the crystal to line of sight) and mounted on a slide 2 mm thick, rubbed with fine sandpaper for better adhesion

The time of complete hardening of epoxy resins can be as short as 1 day or as long as 3 weeks (Schultz, 2001; Garcia-Donas et al., 2017). In the resin Magic Crystal 3D it amounts to 1–2 days (the supplier data), roughly the same as in acrylic adhesives for glass. Curing of epoxy resins can be accelerated by heating which also increases degree of cross-linking of the molecules (Glauert & Lewis, 1999).

At the stage of hardening, epoxy resins have two advantages over acrylic adhesives: insensitivity to atmospheric oxygen and several times lower shrinkage. Their typical shrinkage is 3–5%, and about half of it occurs in still liquid state (Clements, 2006; Horie, 2010). The resin Magic Crystal 3D shrinks by 3% (author's measurement), and some specialized brands show 2% (Glauert & Glauert, 1958; Hand, 2013) and even less (Woods & Stirling, 2019). Like acrylic adhesives, epoxy resins can continue slight deformation for a long time after curing (Mollenhauer, 1993).

Cured epoxy resins, like acrylic UV adhesives, are usually cross-linked thermosetting polymers that cannot flow, melt or dissolve in ordinary solvents (although they can be dissolved by destruction of chemical bonds – however, this requires quite aggressive reagents: Mayor et al., 1961; Litwin, 1985; Neuhaus et al., 2017). Water resistance of epoxies is generally good, although many brands swell slightly under the action of water (Mollenhauer, 1993; Nakka, 2010). Regarding chemical resistance, these resins are generally superior to UV adhesives (Clements, 2006).

Hardness of the cured resin is almost the same as that of the acrylic adhesive: 61 units of Shore D scale (the supplier data), which turned out to be fully sufficient for making ground sections.

Adhesion of epoxy resins to glass is not always stable. The author's practice shows that some sections glued by the resin (including glued to frosted glass) peel off over time in the order of 1 year. This disadvantage of epoxy resins of various brands is also described in the literature (Horner & Lamm, 2011; Woodward et al., 2011; Lamm, 2013; Haas & Storå, 2015; Garcia-Donas et al., 2017). It may be related to the high elastic modulus of epoxies, i.e., appearance of strong stresses due to even small deformations caused by shrinkage or changes of temperature (Davison, 2003). If the resin contains solvents or plasticizers, shrinkage and exfoliation can also be caused by their gradual loss (Horie, 2010). To some extent, epoxy resins can also deform due to absorption of atmospheric moisture. Different brands are able to absorb it in the amount of <1% to 4-5% by weight, and their volume increases by the same amount (Nakka, 2010; Ahmad et al., 2011). Water can also weaken the epoxy-glass bonding directly, diffusing through the epoxy to the boundary and accumulating there. Moisture absorption of epoxies sharply increases and adhesion to glass decreases after onset of equilibrium with air having humidity of >70% (Lefebvre et al., 2000; Neuhaus et al., 2017). Central areas of a thin section located between glasses should reach the equilibrium with air over years, but the peripheral areas are penetrated by moisture much faster (Chang et al., 1997).

The refractive index of the tested epoxy resin, measured using the Duc de Chaulnes method (ratio of resin and hardener was 10:6 by volume; 8 days after curing; vacuumed before curing), is 1.58 ± 0.02 (according to the supplier, 1.5). This is very close to the value for recent bone (1.55–1.56), and therefore the bone becomes almost transparent except for poorly impregnated areas, which become very prominent. A high refractive index is usual for epoxy resins, although it differs greatly from brand to brand. Most often it lies in the range of 1.550–1.585 (Tennent & Townsend, 1984) or, according to the other data, 1.50–1.56 (Cady et al., 1986; Su et al., 2002), but in epoxies used in microscopy it varies at least from 1.49 to 1.65 (Punge, 2009).

No microcrystals in Magic Crystal 3D resin, unlike Loxeal 30-23 adhesive, were observed. However, some epoxy resins used in microscopy are reported to contain square inclusions about 100 μ m in size, which are invisible in ordinary light but shine brightly between crossed polarizers. These inclusions occur in resins of various brands, originate from the hardener and can be eliminated by heating to 50 °C (Heck et al., 2020). Some authors have noted in epoxy resins droplet-like inclusions 3–

 $6 \,\mu\text{m}$ in size, the appearance of which can be prevented by heating (Nye et al., 1972).

A common disadvantage of epoxy resins compared to acrylics is the fluorescence stronger by two orders of magnitude (Punge, 2009), which impedes fluorescence microscopy. However, fossil bones are not often examined by this method.

The major disadvantage of the tested epoxy resin is strong birefringence in the sites of deformations (Fig. 9). It appears, in particular, in the pores and near the surfaces of the bones (apparently from the shrinkage of the resin during hardening, despite 3 times lower shrinkage than in the UV adhesive), as well as in scratches from grinding. This property of epoxy resins can also be seen in microphotographs from the literature (e.g., Schultz, 2012; Wilson & Chin, 2014).

Properties of PMMA solution and cyanoacrylate

Two other readily available media were also tested for embedding: a solution of acrylic glass (poly(methyl methacrylate), PMMA) in acetone and cyanoacrylate adhesive ("Sekunda"). PMMA is a linear polymer, therefore it is soluble in some liquids. Cyanoacrylates are a class of compounds that can give both linear and cross-linked polymers; usually their chains are quite strongly interlinked, so the polymer cannot dissolve without breaking chemical bonds (Davidson & Alderson, 2009).

Cyanoacrylate has been used in a number of studies for making ground sections and other microscopic slides, but did not gain high popularity, although some authors report good results (Liu et al., 2010; Vangala et al., 2016). Its main advantages are wide availability, safety, rapid hardening, low viscosity and, as a result, good permeability, and the disadvantages are significant shrinkage, underfilling of cavities, chemical instability and fragility (Beauchesne & Saunders, 2006; Clements, 2006; Down & Kaminska, 2006; Davidson & Alderson, 2009; Horie, 2010; Haas & Storå, 2015; Garcia-Donas et al., 2017; Neuhaus et al., 2017). Some authors also noted its tendency to crack (Tulander, 2018), to form bubbles (Wells, 1989), occasional presence of crystalline inclusions (Marks et al., 1996) and discoloration of iron-bearing fossils (Howie, 1984; Davidson & Alderson, 2009). General stability and resistance to high and low temperatures in cyanoacrylates is worse than in acrylic, epoxy and urethane polymers. The question of long-term stability of cyanoacrylates remains poorly studied (Down & Kaminska, 2006; Davidson & Alderson, 2009). According to the author's observations and some literature data (Boyde & Bromage, 2021), the major disadvantage of cyanoacrylate adhesive is the appearance of very strong birefringence during hardening.

PMMA, precipitated from solution, is notable for lack of significant birefringence (Fig. 10). But its solution is quite viscous and shows a large shrinkage, which causes appearance of cavities in the pores of the sample. This problem is typical for substances which solidify due to solvent evaporation (Davidson & Alderson, 2009). In addition, neither PMMA solution nor usual cyanoacrylate adhesive can give reliable bonding to glass slides, and gluing to PMMA slides is problematic due to their tendency to warp in contact with the solution and to crack in contact with cyanoacrylate. However, gluing to the glass can be done with specialized adhesive. It is also noteworthy that PMMA exists as cast or extruded sheets which have different properties. If PMMA is used as a slide, cast sheets should be used (due to lack of birefringence), and if it is used for solution preparing, extruded sheets are better (due to smaller size of the molecules and, consequently, less viscous solution). These sheets can be distinguished by birefringence.

Thus, both cyanoacrylate adhesive and PMMA solution proved to be hardly suitable for embedding. It should be noted that PMMA when obtained from monomer rather than solution gives better results and is widely used for recent and sometimes fossil bones, although it still has certain disadvantages (Sanderson, 1995; Schultz, 2012). Cyanoacrylate cannot be recommended for embedding samples which demand high quality.



Fig. 9. Thin sections of diaphysis of tibiotarsal bone of recent chicken (made from adjacent slices of one bone, approximately 0.5 mm apart): a – embedded in UV-curing adhesive for glass Loxeal 30-23; b – in epoxy resin Magic Crystal 3D; c, d – the same samples under polarizing microscope (equivalent of view between circular polarizers, see Fig. 5); size of each image is 1.5×2.0 mm; the sections are approximately 50 μ m thick; mounted on a frosted glass 1.9 mm thick; epoxy resin shows two disadvantages compared with acrylic adhesive; firstly, the resin due to high refractive index, close to that of the bone, makes the bone rather transparent, and in usual light badly impregnated regions become the most prominent features; moreover, the resin leaves more such regions than the acrylic; secondly, the resin shows high birefringence in many places, damaging the image under a polarizing microscope; this birefringence apparently results from deformations caused by shrinking of the resin during curing (seen near the edges of hard objects, especially in vascular canals) and by scratching during grinding



Fig. 10. Thin section of diaphysis of tibiotarsal bone of recent chicken, embedded in acetone solution of poly(methyl methacrylate) and glued to poly(methyl methacrylate) slide; above: usual light; below: polarized light imaging in the same way as in Figure 9; the size of each image is 2.3×1.6 mm; polymethyl methacrylate obtained from the solution shows almost no optical activity, has an acceptable refractive index and penetrates into recent bone well enough; nevertheless, the perspective of its use is limited by a number of disadvantages, including high shrinkage

Embedding technique

The container for embedding should be large enough to leave an approximately 1 cm wide space all around the sample. It will help to obtain a flat and unscratched sample surface during grinding. A convenient container can be made from part of a polyethylene syringe with sealed nozzle. After curing, such container can be easily dissected and detached from the embedded block: adhesion of polyethylene to virtually all materials is very weak.

Proper position of the sample in the container can be achieved by its fixation in a tripod made of thick polyethylene from food package (Fig. 11).

Embedding liquids best impregnate samples in the case of embedding in vacuum and subsequent increase of pressure. Vacuuming also gives another advantage: removes dissolved gases from the liquid, increasing its ability to dissolve bubbles. In the case of UV-curable acrylics, removal of dissolved oxygen also should enhance curing.

The container with the sample and container for the embedding liquid must be affixed in the vacuum chamber in such a manner that the liquid would spill into the sample container when vacuum chamber is tilted (Fig. 12). The containers can be affixed with plasticine, which must be taken with excess, because it tends to detach if wetted with acrylic adhesive. If significant volume of the liquid is utilized ($\geq 1 \text{ cm}^3$), it is useful to put in its container several boiling chips, which would facilitate degassing and enable it to be observed. Freshly cracked grains of hard stone or porcelain 2–3 mm big are suitable as boiling chips. Finally, the embedding liquid must be poured into its container, the vacuum chamber must be closed and the air must be pumped out.



Fig. 11. Sample of fossil bone secured in a polyethylene tripod (with a millimeter ruler) and the same sample after embedding and curing



Fig. 12. Embedding of a sample in vacuum: after pumping the air out, the jar is tilted and the embedding liquid spills into container with the sample *Biosyst. Divers.*, 2023, 31(1)

The adhesive can be degassed by vacuuming during 2 hours under ~ 0.02 atmospheric pressure in darkness. It can be useful to heat the adhesive with an incandescent lamp just before the embedding for decreasing its viscosity.

For embedding, the vacuum chamber must be slowly tilted to make small amount of the liquid reach the sample, touch one of its sides and begin infiltration. The sample should be fully covered with liquid not earlier than when it finishes soaking; remains of air must have an exit.

After embedding, pressure must be raised back to atmospheric pressure or, if possible, higher. It would cause shrinkage and better dissolution of air remnants in pores. Before curing, it may be reasonable to wait a few days and facilitate dissolution of these remnants by changes of pressure and (or) temperature, which will cause changes in their volume and, consequently, circulation of embedding medium in the pores. The sample must be kept in darkness and, preferrably, in an oxygen-free environment (for example, the chamber can be filled with the gas from a lighter).

Curing

UV-curable adhesives can be cured with the help of both widespread types of UV lamps: fluorescent lamps with radiation wavelength 365 nm and LED lamps with 395–400 nm. In close proximity to the lamp, the adhesive solidifies during seconds or tens of seconds, but full-fledged curing, according to instructions, requires irradiation during several minutes. It should be borne in mind that:

-during curing, the adhesive shrinks by 9% and strongly heats up;

- transparency of the adhesive for UV radiation is low: 1 cm thick layer is almost opaque.

Consequently, the curing must progress from the bottom upwards. In the beginning, the entire container except the bottom must be shadowed by opaque material, and then this shield must be slowly raised. If irradiated from all directions, the adhesive will solidify firstly in the outer layer, and the inner part will suffer rarefaction, which causes growth of air bubbles and their sucking from outside.

Heating during curing causes evaporation of the adhesive. Therefore, it must be cured under good ventilation or in sealed container. After curing, at least one day must pass for acquisition of final hardness and termination of most shrinkage and deformations.

Grinding

Grinding and polishing of the sample surface can be done on grinding machines (Wilson, 1994; Cho, 2012; Lamm, 2013) or manually, e.g., with sandpaper lying on a flat hard surface. The manual method does not require special equipment, is more gentle and allows better control over the process (Horner & Lamm, 2011). It is discussed below.

Grit size is chosen according to required amount of grinding, starting from the coarsest and ending with the finest one. Fragile, in particular fossil, samples should be ground only by fine sandpaper: not coarser than P400, and preferably even P1000.

Median particle size is approximately inversely proportional to the number in sandpaper designation according to Federation of European Producers of Abrasives (FEPA) and ISO standard. For instance, the designation P400 corresponds to median grain size 35 μ m, P1000 – 18.3 μ m, P2500 – 8.4 μ m (ISO 6344-1:1998).

Another important property of the sandpaper is the mode of coating: open-coated (if the particles cover the surface incompletely) or closecoated. Open-coated sandpaper is not prone to clogging with clumps of waste and, therefore, can be more effective.

Some brands of sandpaper are prone to grit shedding. They are virtually unsuitable for the work, but their ash can be used for frosting of glass (see below).

Fine sandpaper quickly becomes clogged, littered with lumps of swarf and wom out. Its workability can be restored to some extent by cleaning with a vacuum cleaner through a fine metallic grid (it should be also done before first use of the sandpaper). Lumps of swarf can be removed by a hard brush or a knife. The cleaning would be more complete if before the usage the sandpaper is greased with a small amount of graphite or charcoal which loosen the waste (although diminish grinding efficiency). Water-resistant sandpaper can be washed by a brush with soap and dried between hard surfaces to prevent warping.

Efficiency of the sandpaper (amount of material which it can remove before loss of workability) can be characterized by thickness of removed material if evenly distributed on the sandpaper surface (Duwell, 1986). This thickness equals the removed volume divided by area of sandpaper. Experiments show that the efficiency depends on median grain size nonlinearly (Fig. 13), and in the case of coarse grit (P400 and smaller numbers) becomes highly variable and difficult to determine. No difference of sandpaper efficiency for epoxy resin and acrylic adhesive was observed.



Fig. 13. Efficiency of silicon carbide sandpaper (during the first use) vs its median grain size: at the size 35 μ m (P400) the efficiency begins to vary with the brand heavily (from <3.8 to >15 μ m)

This data allows choosing of optimal grit number. A sheet of the standard size 23×28 cm and designation P600 during the first use would remove from a wafer of usual size (5 cm²) a layer approximately 500 µm thick, with designation P800 – 300 µm, P1000 – 200 µm, 1200 – 100 µm, 2000 – 20 µm. A glass sheet of the same size, frosted using ash from sandpaper P1500 (see below), has efficiency 0.005-0.010 µm and would remove from such a wafer 0.5–1.0 µm.

Grinding with sandpaper creates a slightly convex surface (with convexity tens of micrometres high). In addition, the surface can acquire relief due to different hardness of the sample and embedding medium. For obtaining a flat surface, the last phases of grinding should be done with a hard grinding tool. A ground and frosted sheet of thick glass is suitable. It can be covered with abrasive paste (Reed & Mergner, 1953; Pawlicki, 1978), but clean glass grinds more gently and does not contaminate the sample. The surface of the glass can be flattened and frosted by grinding against another glass with addition of wet ash from wom sandpaper P1500–P2500. During first processing of the glass, it must be repeated till vanishing of visible imperfections. The abrasive must be free from grains substantially larger than average, otherwise the frosted glass would scratch samples. Large grains can be removed by agitation of the abrasive in water, where they sink during ~5 seconds. After processing of the glass, the abrasive should be washed away while wet.

Frosted glass quickly loses grinding ability and should be re-frosted frequently. So, a large sheet (at least $\sim 10 \text{ dm}^2$) is preferable.

The sample should be ground by circular motion with weak pressing and slow moving to new areas of sandpaper or glass. Again, there must be no abrasive grains significantly larger than average (grains that make significant scratches are easily spotted by touch and must be avoided). If the grinding did not leave outstanding scratches, the abrasive can be replaced by a finer one.

Requirements for grinding quality depend on embedding medium. Microscopic scratches on acrylic adhesive will vanish without any trace after gluing of a coverslip, but scratches on epoxy resin will remain well visible under polarizing microscope. However, it is better to wipe out even microscopic depressions to prevent preservation of abrasive powder and to remove zones of microdeformation of the sample (Nye et al., 1972). Before mounting to the slide, it can be useful to re-impregnate the sample surface with the adhesive and cure it. It fixes (at least partly) the problem of possible under-impregnation of sample interior, which can cause numerous flaws (opacity, fragility, microbubbles in pores and ability to absorb liquid adhesive, which induces bubble growth during mounting). Wetting of the working surface with the adhesive should be done in vacuum as described above. Afterwards, the sample must be removed from the vacuum, excess of the adhesive must be squeezed out by pressing the working surface against flat glass through transparent plastic film, and the sample must be cured with UV irradiation through this glass. Then the surface should be finely ground again to be precisely flat and clean.

Mounting

The most widespread microscope slides are 1 mm thick. Unfortunately, they are prone to breakage during insufficiently careful grinding and accompanying work, especially if frosted. For ground sections of valuable objects, 2 mm thick slides can be advisable. They impose some limitations on illumination of the sample, but at low magnifications (objectives 4x– 8x) which are still adequate for many objects, including bones, it does not worsen the image noticeably, especially if flat-field correction is applied. Before mounting, the thickness of the slide in the working place should be measured for subsequent determination of the section thickness (thickness of the slide in different places can vary by tens of micrometers).

The slides can be frosted for improving adhesion (Lamm, 2013), although the tested adhesive for glass is fully compatible even with non-frosted slides (no troubles were observed after 3 years). Frosted surface can be obtained by grinding the slide against a larger glass with the addition of ash from burnt worn sandpaper P1000–P2000. Additionally, it ensures a flat surface.

The sample must be mounted soon after grinding: the same day, because embedding medium can undergo microscopic shrinkage over a long time (at least weeks) after curing, deforming the ground surface.

It would be helpful to attach a plasticine handle to the back side of the sample. It greatly facilitates accurate apposition of the sample to the slide and decreases probability of faults. The tip of the handle should be melted beforehand for attaching without pressure.

The main dangers during the mounting are dust and air bubbles. Most particles of domestic dust are strongly birefringent and spoil the image in polarized light. Additionally, samples can contain unfilled pores littered with abrasive during grinding. Therefore, immediately before mounting, the sample must be cleaned with a thin-haired brush or piece of felt and checked under a microscope. For better visibility, mounting should be done with a bright light and black background. Air purifier is desirable.

The slide should be carefully washed, rinsed with distilled water, quickly dried over a fire and placed working side down, but not touching anything by the central part. Just before gluing, the slide should be heated by several dozen degrees for removing invisible water condensate.

The first 1–2 drops of the adhesive should be thrown away: they can contain bubbles, dust and increased oxygen content. Then the adhesive must be dropped on the slide (but not on the gluing place) and on the wafer with embedded sample (but not on the sample itself). For preventing formation of attached bubbles, the adhesive should be allowed to gradually crawl on the target places by tilting the slide and the wafer. If the sample still has unfilled pores, it should be done in vacuum. After the sample and slide are wetted with adhesive, they should be slowly attached to each other starting from one edge for allowing possible bubbles to come out. After joining, the contact should be checked with the help of magnifying glass.

During mounting, acrylic adhesive must stay in the form of a thin layer as briefly as possible for avoiding crystallization of its compounds due to evaporation of volatile constituents (see above).

Before curing, the sample should be weakly and uniformly pressed to the slide (e.g., with a load of \sim 10 grams), otherwise it is prone to nonparallel placement. Compensating of this non-parallelism during grinding is much more difficult.

While the sample remains thick, it can be useful to photograph its polished side (after or before mounting). Visibility of some details (e.g., pattern of vascular network and secondary osteons in the bones) is much better on thick sections than on thin ones (Fig. 14), although thick samples are not suitable for polarized light microscopy.



Fig. 14. Fossil bird bone (transverse section of humerus, cf. *Dasornis*, Eocene of Ikove, Ukraine): thin section in transmitted light (upper) and thick section in reflected light (lower); the lower image is horizontally reflected to match the upper one; advantages of thick sections are seen, including better visibility of vascular network pattern and secondary osteons

Grinding of the mounted sample

After curing of the adhesive, the mounted sample should be ground from the reverse side similarly to the first grinding. If the mounting medium has a sticky surface, like UV-curing acrylic adhesives, this sticky layer must be removed, otherwise it will catch and release abrasive grains, which will scratch the surface. Non-hardened adhesive can be erased with the help of ethanol or soapy water – as fast as possible, because both ethanol and water somewhat soften cured medium.

In the last stages of grinding, thickness of the sample and its uniformity must be controlled with the help of a micrometer or microscope. It should be kept in mind that covering with adhesive during gluing of coverslip will make the sample more transparent.

Optimal thickness of the section depends on the object. For fossil bones it usually constitutes, according to author's practice and most literature data (Varricchio, 1993; Schultz, 2001; Garcia-Donas et al., 2017; Sellés et al., 2021), approximately 70 µm. However, transparency of fossilized bones varies, so there is no standard thickness of paleohistological sections, and used values range at least from 30 µm (Monfroy & Kundrát, 2021) to 100 µm (Monfroy et al., 2021). For recent bones, standard thickness exists for study of collagen fibers orientation: $100 \pm$ 5 µm (Bromage et al., 2003; Warshaw et al., 2017), although study of cellular components requires just 4-6 µm thick sections (Kang et al., 2003). Thickness of 100 µm is also often employed for microradiography of both recent and fossil bones - imaging using X-rays and photographic film directly pressed to the section (Schultz, 2001). For the majority of geological samples, the standard of 30 µm is employed from the beginning of petrography (Miller, 1988; Worley, 2009). This appears to be also the optimal thickness of eggshell (whose transparency is lower, and birefringence higher than that of bone). Some authors have even suggested a thickness of 2-12 µm for carbonate samples (Lindholm & Dean, 1973; Green, 2001). Although thickness of the sections is often not specified in publications, it is worth mentioning, because it does matter for quantitative investigations and comparison of different objects (Bromage & Werning, 2013). If the last phases of grinding or polishing have to be delayed, samples should be kept in a container with a desiccant (anhydrous calcium chloride etc.), because cured acrylic adhesives can slowly absorb atmospheric moisture and somewhat soften.

Coverslip attaching

Coverslips are not always used in paleohistology: they are omitted, in particular, if the sample is planned to be studied by methods requiring direct access (Miller, 1988; Wells, 1989; Green, 2001; Lamm, 2013). But the coverslip protects the sample, and the adhesive which attaches it reduces visibility of scratches and increases transparency of the sample (sometimes this is achieved by covering with the adhesive alone, but such covering has several drawbacks and is not recommended for permanent mounts: Neuhaus et al., 2017). In particular, samples should be protected from water vapor due to some hygroscopicity of certain mounting media (including acrylic adhesives) and due to ability of water to diffuse through any polymer medium and weaken its adhesion to the microscopic slide (Chang et al., 1997; Davison, 2003; Neuhaus et al., 2017). In addition, microscope objectives are often designed for use with coverslip of standard thickness.

Attaching of a coverslip is made similarly to attaching the sample to a slide, but now full-fledged microscopic control of cleanness becomes possible. The main danger is incorporation of dust and air bubbles. Clean air, bright illumination and black background help preclude this.

The adhesive should be dropped near the embedded object (after discarding 1–2 first drops) and allowed to crawl onto it. The adhesive should be taken with excess, otherwise the coverslip tends to stick to the object with development of bubbles. One edge of the coverslip must be placed on an adhesive-wetted surface, and then the entire coverslip must be slowly put down, supported with a needle or another thin instrument. Before curing, the condition of the microslide should be checked under the microscope. Curing should be done with uniform and initially not very intense UV illumination to prevent strain caused by shrinkage of the adhesive.

Safety considerations

Hazards in the field of thin section preparation include vapors of embedding/mounting liquids, possibility of contact of these liquids with skin, dust from grinding and, to some extent, UV radiation, if used for curing.

Constituents of the uncured adhesive Loxeal 30-23 (2-hydroxyethyl methacrylate, isobornyl acrylate, acrylic acid, diphenyl (2,4,6-trimetylbenzoyl) phosphine oxide) have irritating and allergenic effect; the last 3 are qualified as toxic to aquatic life (Gerrits & Horobin, 1996; Foti et al., 2015; Aerts et al., 2020; PubChem database). According to the manufacturer, the adhesive Loxeal 30-23 causes skin irritation and serious eye irritation, may cause an allergic skin reaction and respiratory irritation, is very toxic to aquatic life with long lasting effects. If on skin, it must be washed with a large amount of soap and water; if in the eyes, they must be rinsed carefully with water for several minutes. Acrylic monomers are volatile (Glauert & Lewis, 1999), and the adhesive releases significant amount of vapor due to heating resulting from curing. Its vapor can be detected by the characteristic odor (reminiscent of pine resin).

Irritant effect, in particular the ability to sensitize skin or to cause allergic dermatitis, is common for monomers of (meth)acrylic media. Methyl methacrylate has also a neurotoxic effect due to its ability to dissolve lipids. But toxicity of (meth)acrylic monomers is low: their LD₅₀ for mammals amounts to several or several dozen g/kg in most cases (Ellis, 1989; Tobler & Freiburghaus, 1990; Gerrits & Horobin, 1996; PubChem database), although auxiliary components of embedding media may be more toxic (Glauert & Lewis, 1999). Substances similar to acrylic mountants are used in medicine, in particular dentistry (Ellis, 1989). Particularly, the polymer of hydroxyethyl methacrylate is known to be safe for living tissues and is widely used for biomedical purposes.

Epoxy resins require more careful handling: they show to some extent not only irritating and allergenic, but also mutagenic properties (in some components of certain brands, according to animal experiments, up to carcinogenicity). Low viscosity resins are more harmful (Ringo et al., 1982, 1984; Ellis, 1989; Mollenhauer, 1993; Glauert & Lewis, 1999). Hardeners of epoxy resins, especially amines, which are the most common, are also more or less toxic (Borgstedt & Hine, 1988; Ellis, 1989). Polyester resins have irritant and other harmful properties as well (Ellis, 1989, 2003a).

Work with all the above-mentioned media must be conducted under good ventilation and using impermeable and non-slippery gloves. If contaminated, gloves must be changed, because many monomers of these media still permeate many models of gloves, including laboratory ones, during minutes or tens of minutes. Contaminated items should be discarded or isolated immediately to prevent evaporation of the liquids. In the case of skin contamination, it should be washed with cold soapy water (Ringo et al., 1982, 1984; Borgstedt & Hine, 1988; Tobler & Freiburghaus, 1990; Ellis, 2003b; Henriks-Eckerman et al., 2015).

Long-wave ultraviolet radiation (UVA), used for curing, is weakly hazardous, but nevertheless can negatively influence the skin and eyes (WHO International Programme on Chemical Safety: Environmental health criteria 160: ultraviolet radiation). In addition, some lamps can give some amount of harder radiation (Philips, 2020). Therefore, protection from UV radiation, e.g., good sunglasses, is advisable.

During grinding, a respirator is necessary for protection from dust. This is especially important for the work with epoxies, as they usually contain residues of unreacted monomers (Mollenhauer, 1993; Borgstedt & Hine, 1988; Ellis, 1989). If dry frosted glass is used for grinding or polishing, immediately after use it must be moistened from a spray can (spraying above the glass and not on the glass itself) for immobilizing glass dust, and then washed with a brush.

Conclusion

Non-specialized UV-curable acrylics can be successfully used for embedding hard and friable biological, geological and other samples with various optical and mechanical characteristics for microscopic investigation. These media are notable for negligible birefringence, making them well-suited for polarized light microscopy. Besides this, they are rather easy to use and have a number of other advantages, but, like other embedding media, have specifics which should be kept in mind to obtain the best results. Similarly to embedding, other steps of ground section preparation can be successfully done using only easily accessible tools and supplies.

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