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DOES THE EMBEDDING CHEMISTRY INTERACT WITH TISSUE?

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Abstract

Resins and resin compounds interact with tissue in two ways, physico- chemically and by chemical reaction. The physico-chemical influences affect both the structure and biological activity of the tissue causing proteins to change shape or phase equilibria to be disrupted by a change in solvent or by the growth of polymer networks within existing biological polymeric structures. Chemical reactivity between tissue and resin components also reduces biological activity by changing either the structure of polymeric matter already present, e.g., crosslinking proteins, grafting hydrophobic resins onto hydrophilic protein backbones, or by modifying the hydrophobicity of specific sites, e.g., acylation of amino groups by hydrophobic anhydrides. The consequences of all these interactions can only be a fall in activity of the tissue leading to longer reaction times and the need for amplification of specific group reaction. Low crosslink densities, moderate temperatures, and partial rather than total removal of water are possible ways of reducing the effects of resin tissue interaction.

Key Words: Protein, Carbohydrate, Lipid, Epoxy, Anhydride, Acrylic, Phase Separation, Peroxide, Tissue Interaction, Interpenetrating Polymer Networks.

Introduction

The embedding of a piece of tissue is such a multifactorial act that even though sound arguments can be advanced to support interaction mechanisms between tissue and embedding resins, quantification of the influences of such interactions is very difficult. However, what can usefully be discussed are the mechanisms of such interactions and the conditions under which such interactions become unfavourable. The interactions are of two basic types, one a chemical reactivity of the embedding resin system acting as reagent and the tissue as substrate, and the other the physicochemical consequences of solvent/solute, monomer/polymer, and polymer/polymer interactions. Both types of interaction can be present in the same system and often influence each other, especially during phase changes and phase separation. In this respect the nature of the tissue is of great importance. Fixed tissue can be regarded as a three-dimensional co-polymer network in which normally polymeric species, proteins, carbohydrates, etc. are joined to structures that are normally fluid: e.g. lipid bilayers, which in the fixed form may be rigid crosslinked systems. Unfixed tissue will have fluid membranes capable of diffusion and hydrophobic displacement when juxtaposed to resin rather than aqueous species. Hence when considering interactions the overall polymeric structure of the tissue is important in determining the thermo- dynamic consequences of introducing a resin solute. Finally, the hydration state of the tissue can have a great influence. Tissue exists in a state of almost maximal hydration; dehydration prior to resin infiltration or by the resin itself can change the electrostatic and hydrophilic character of the environment through which resin must diffuse and with which it will interact.

Since microscopy relies on a careful identification of structures, adsorbed dyes and markers, resins should cause as little distortion of structure as possible and leave the greatest number of sterically undisplaced reaction sites on the substrate as possible. It is as a guide to maximising these criteria that this paper is written.

The physico-chemical interaction of embedding resins and tissue

Assuming that the tissue has not been over-fixed and that its crosslink density prior to embedding will allow the reagents of later staining techniques to enter the tissue, the resin has several potentially troublesome physico-chemical interactions that can either reduce apparent antigenicity or enzymatic activity of tissue, or cause artifactual changes.

Protein-solute interactions

Proteins that have either antigenic or enzymatic activity also possess very specific structures. The precise dimensions of those structures can be an influence on the activity of the protein (Eagland, 1975). However, such proteins rely on a careful balance of hydrophobic forces to maintain such structures. Haemoglobin is a case in point; it is a molecule structured like a catcher's mitt, the adsorbed gas being the ball. Place haemoglobin in water containing alcohol and the "mitt" opens (Cordone et al, 1982). Less than 5% of alcohol is enough and its oxygen carrying capacity is impaired. Place enzymes and antigens in resin rather than water, then cure the resin in situ:- rehydration of the tissue and resin combined is unlikely to return the tissue protein to its original size and shape. An additional problem can arise from the presence of free monomer. When a monofunctional monomer has been used as the basis of an embedding resin, and under the mild conditions of cure that prevail in microscopy, a fair proportion ($\sim$ 10%) of the monomer will not polymerise but remain in the block as a free solute. On rehydration of the tissue during the staining procedure only part of this free monomer may be extracted by the water, leaving the remaining monomer to interfere with the activity of the protein in a similar manner to the alcohol referred to earlier. To minimise such problems, either allow longer for free monomer to wash out of the section or, in the case of monomers with limited aqueous solubility, a co- solvent such as alcohol or acetone could be used to remove free monomer prior to staining.

Phase separations during polymerisation

Polymerisation is by definition the creation of polymer in a volume where once there was only monomer. This phase change is capable of causing other species present in the system to precipitate. The polymerisation process itself is rarely isothermal and may involve raising the temperature by anything up to 100°C. This makes possible the locking of active compounds in their high temperature structures, for as the polymers form, the internal viscosity of the entire system rises and diffusion rates fall. Such changes will have little effect on the microscopic appearance of proteins and carbohydrates because protein and carbohydrate that were in a water soluble state will have been either precipitated or lost during dehydration and those species in an already condensed form will have changed in size and conformation during resin infiltration due to the removal of water from the tissue. However,

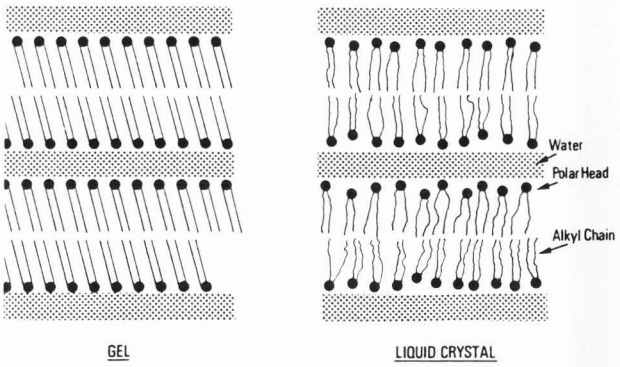


Fig.1 Phase transition in lipid bilayers.

the lipid may still be soluble, though in a much changed molecular arrangement. When polymerisation begins there are many changes that can occur in lipid structure. Temperature changes can bring about changes of degree of crystallisation producing mixtures of liquid crystals (Fig.1). This is particularly true for hydrophilic monomers where not all the water is removed during dehydration, leaving water contents of 5% or more in the tissue. Under such conditions, mesomorphic lamellar structures can form as the temperature rises. This behaviour varies with the lipid concerned, but cases have been reported of such transitions occurring in 5% water/lipid mixtures at temperatures between 40°C and 80°C (Chapman & Wallack, 1968).

The lipids can be further influenced in their structure by the polymerisation if the monomer is also the solvent in which their micelles are formed. As the polymer content rises so the lipids are thrown out of solution and if this occurs at a temperature above that required to form lamellae the necessary molecular mobility for the lipids to revert to the gel state more commonly found at phsyio- logical temperatures. may well be lost upon gelation of the final cured block. The significance of such "frozen" transitions caused by solvents being polymerised is that features reported in resins cured at high temperatures may not be found in resins cured at low temperatures and should be borne in mind when lipids are known to be present.

Interpenetrating polymer networks

If we consider the polymers present in the tissue infiltrated by uncured resin, we can envisage two types of structure: amorphous polymer regions containing some monomer, and crystalline highly ordered condensed polymer regions containing monomer, all crosslinked by glutaraldehyde or formaldehyde. Upon polymerisation the infiltrated monomer will form a crosslinked matrix that is within the orignal crosslinked matrix, an interpenetrating polymer network (Fig.2). Thermodynamics predict that the heats of mixing of the two matrices are too small to prevent phase separation and they should duly separate (Flory, 1953). However, because

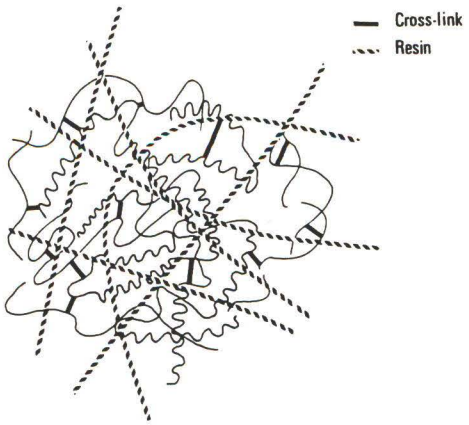


Fig.2 An interpenetrating polymer network of crosslinked protein and a resin.

both matrices are crosslinked, this is a physical impossibility. This ploy is used in industry to make very tough polymers, the toughness arising from the thermo- dynamic metastability (Sperling et al, 1970). However, in our biological interpenetrating polymer network it can be a disadvantage because when we rehydrate the system in order to demonstrate antigens, enzymes, etc. the highly stereo specific proteins and carbohydrates are under strain and held in unnaturally close proximity to resin molecules with which they are incompatible. This must greatly reduce their activity for not only is access to antigenic sites restricted, but the sites themselves are distorted. Hence, where antigenicity and enzyme activity are important, the lower the crosslink density of the resin the better and the lower the number of fixation crosslinks also. In this way the formation of interpenetrating polymer networks is reduced and easier access to active sites is maintained.

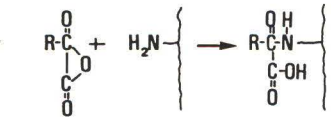
Direct chemical reaction between tissue and embedding resin.

There are many ways in which the components could, under the right temperature conditions, react with tissue either in its dehydrated or anhydrous state. However, many reactions would occur so seldom that no effect would be detectable at the microscopic level. The reactions described, therefore, are thought to occur easily under normal curing conditions, although some are avoidable if curing conditions are controlled carefully.

Reactions with proteins

The most common reactions between resins and proteins occur at sites rich in nucleophilic substituents and end groups (Table 1).

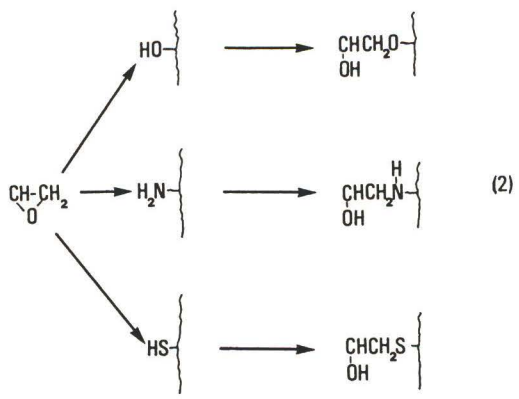
Acylation of protein nucleophiles by anhydrides has been described several times (Putnam, 1953). It is known to be possible to acylate amino, hydroxyl and tyrosine groups with better than 90% conversion under both anhydrous and aqueous conditions using the same long chain anhydrides as those used in epoxy resin curing systems (Eq.1). Best conversions are obtained at pHs 7-8 and at temperatures between 0-25°C. The reactions proceed quickly, requiring less than 20 minutes exposure. This is an interesting reaction when considered with the points made previously about hydrophobic balance in active proteins. Consider the consequences of replacing a hydrophilic amino group with a hydrophobic long alkyl chain. The effect will be reduced greatly by the competition for anhydride by the epoxy groups. However, the latter reaction is slow at room temperature and, antigens and enzymes will therefore be at greatest risk from acylation reactions during infiltration.



(1)

Anhydride Protein

Esterification of proteins is most favoured by acid conditions and hydroxy substituted monomers are therefore unlikely to react. However, epoxy groups are very reactive to proteins (Windmueller et al, 1959). The 1,2 epoxides are not selective in their attack on proteins, being universally reactive to a wide group of nucleophiles (Eq.2). The rate of reaction of the epoxides is thought to be only limited by steric factors and internal viscosity, hence large bulky molecules will react more slowly than smaller less viscous resins (Whitfield and Wasley, 1964). The consequence of these reactions will be a fall in the activity of the protein as extra crosslinks form and hydrophobic forces are modified.

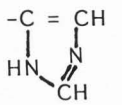
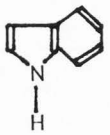


(2)

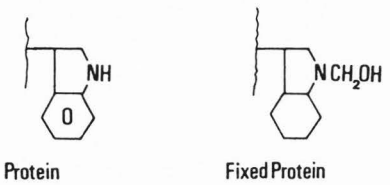
Reactions between carbonyl bonds and proteins are known to occur and form the basis of

Table 1

Nucleophiles present in Proteins

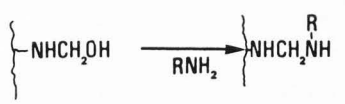
Nucleophiles	Structure	Occurrence in proteins	Reacting group
Carboxyl	-COOH, $-COO^-$	Glutamic acid Aspartic acid Chain ends	epoxy
Phenol	Ph-OH, $Ph-O^-$	Tyrosine	epoxy anhydrides
Amino	$-NH_2$	Lysine Chain ends	epoxy anhydrides
Imidazole		Histidine	epoxy anhydrides
Guanidyl		Arginine	
Indole		Tryptophan	epoxy anhydrides
Amide	$-CONH_2$	Glutamine Asparagine	epoxy anhydrides
Aliphatic Hydroxyl	-OH, $-O^-$	Serine threonine	epoxy anhydrides
Sulphydryl	-SH, $-S^-$	Cystine	acrylic radicals epoxy
Disulphide	- S - S -	Cystine	peroxide UV radiation
Thio-ether	$-SCH_3$	Methionine	acrylic radicals

(3)



Protein Fixed Protein

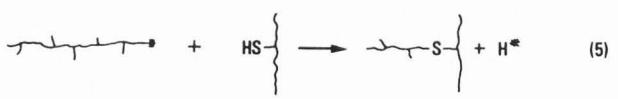
aldehyde fixation (Eq.3 & 4). The melamine/ formaldehyde resins are capable of similar interactions, being capable of reaction to a wide variety of protein substituents (Frenkel-Conrat and Mecham, 1949). Also, formaldehyde fixed tissue is capable of further reaction with the primary amines used as activators in epoxy resin systems. Such reactions have already been used to modify biological function and activity deliberately.



(4)

The effects of peroxides on protein are various and very dependent on conditions; the effect of benzoyl peroxide on protein is probably quite small when it is considered that less than 1% of benzoyl peroxide is needed to cure acrylic resins. However, peroxides are used to remove epoxy resins and these pose a greater threat to protein activity, attacking as they do the -S-S- crosslinks so essential in determining the shape of many active proteins (Elod et al, 1942).

Exposure of protein rich in cystine to radiation has been known to result in some grafting of acrylic and vinyl polymers. However, even when x-rays are used graft efficiency is poor and ultraviolet light is poorer still (Tazuke, 1977). One way in which some grafting of radically polymerised systems may occur is if cure conditions are too severe. In such cases large numbers of radicals are produced and the possibility of chain terminations by interaction with the polymer chain increases - SH groups being particularly efficient (Eq.5). This results as a too highly crosslinked block, poor cutting and staining characteristics and perhaps a fall in the activity of the protein that has been grafted. To avoid such problems one should therefore use the mildest cure conditions that time allows.

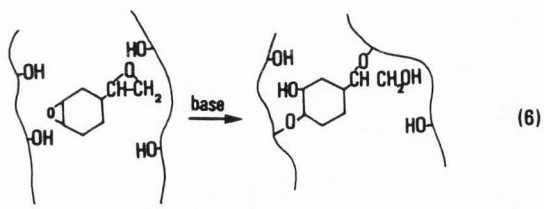


Acrylic Radical Protein Graft

Interaction with carbohydrate

There are many reactions possible with resin components and carbohydrates. The investigations that have produced such information centre on the cotton and paper industries, with polymers or polymer precursors being grafted on to carbo- hydrate backbones to improve wet strength, crease resistance, fire resistance, etc. However, such grafting requires harsh conditions or specific catalysis and one is led to believe that little resin/carbohydrate reaction occurs in preference to the normal curing reactions of the resin.

The epoxy resins do react freely with hydroxy groups on carbohydrates and cyclohexene dioxide (Spurr resin) has been specifically used to improve the shrink resistance of cotton (Reeves and Guthrie, 1964). However, the graft efficiency is small even for this reagent, the most mobile of the epoxies used in microscopy (Eq.6). Paradoxically, it may be a useful interaction for most researchers who, not requiring to carry out any specific stains on carbohydrates, find the improved mechanical properties of the carbohydrate/graft an advantage when cutting sections. However, if specific carbohydrate stains are required acrylics are more likely to reduce activity least.



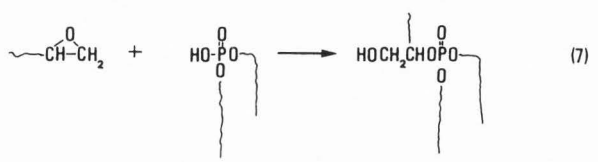
crosslinking of carbohydrates by cyclohexene dioxide (Spurr's resin)

Interaction with lipids

The overwhelming interaction of resin components with lipids is that of extraction during the infiltration stages when in the company of prosolvents such as acetone and alcohol. This is often a blessing, especially in electron-microscopy, because of heavy staining of unsaturated groups by osmium tetroxide, or in the light microscopy of bone where lipid needs to be extracted at some point if it is not to plasticise the cured resin so much that its glass transition temperature is lowered below that of room temperature and the block becomes uncuttable. The embedding resin with the lowest lipid solvent power is L.R.White, which removes so little lipid that bone must be defatted prior to infiltration for best results.

The unsaturation of lipids means that resins that polymerise radically may well graft onto the lipid incorporating as an internal plasticiser in the back bone chain. It is unlikely that peroxide effects the unsaturation in lipid, although long UV cures at low temperatures may cause dissolved oxygen to oxidise the unsaturated alkyl chains.

Epoxides are very reactive to phosphate esters and may well react with membrane lipid to form di and tri esters (Lee and Neville, 1967), although such grafting could well stabilise membranes in the electron beam. Once again, if specific end groups are to be visualised then the less reactive resins are the acrylics (Eq.7).



Epoxy Lipid Epoxy/Lipid graft

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