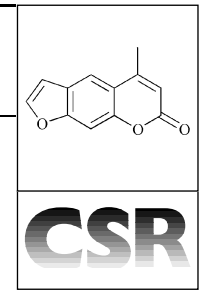
The emerging chemistry of blood product disinfection



Mark Wainwright

University of Central Lancashire, Preston, UK PR1 2HE. E-mail: Mwainwright@UCLan.ac.uk; Tel: 01772 894385

Received 18th July 2001
First published as an Advance Article on the web 23rd January 2001

Given the importance and limitations of the blood supply worldwide, widely applicable procedures for the inactivation of pathogens (viruses, bacteria, protozoa *etc.*) in donated blood and blood products are now required. Conventional treatments such as ultraviolet irradiation cause damage to therapeutic components in the blood, so more targeted approaches are being sought. These include targeted chemotherapy, photochemotherapy and photodynamic antimicrobial chemotherapy (PACT).

1 Introduction

The importance of blood as the major transport vehicle in mammals—literally a life support system—is long established. Given this understanding, it is perhaps not surprising that attempts to rationalise disease as a symptom of 'bad blood' and the use of bleeding (phlebotomy) in its treatment is a logical, if alarming, approach. The converse approach employing donated blood in the replenishment of damaged or diseased patients is equally logical, though this began at the time of the Renaissance and employed animal blood. Since the microscopic differences in the make-up of human and animal blood (typically calves') were not then appreciated, it is not surprising that such transfusions were mainly unsuccessful.

The first transfusion of human blood is believed to have been carried out in 1818 by the British obstetrician Blundell, but the first documented success did not occur until as recently as 1908 when Alexis Carrell transfused an ailing newborn girl using her father's blood.

In addition, early transfusions (successful or otherwise) were fraught with many more dangers than the routine practices of the 21st century. In most of the modern world we are quite blasé about the sight of a sick patient with a hypodermic and tube connecting his or her arm to a suspended 'blood bag'. In Carrell's day transfusion involved the direct transfer of blood from the donor to the recipient, who therefore lay side by side so that an artery (!) of the former could be directly sutured to a vein of the latter. A risky business indeed!

The growth of blood transfusion and thus transfusion services and businesses was rapid after the shock and massive loss of life in World War 1. Major breakthroughs such as the addition of citrate to collected blood to stop coagulation, and the development of reliable, easy to use equipment meant that by the Second World War, major operations such as the D-Day landings could be supported efficiently with replacement blood products. The amount of detailed strategic planning required for such operations laid the groundwork for modern blood services which are so important in the support of a modern nation's health.[1]

1.1 Blood and infection

Just as we understand that blood is responsible for the transport of oxygen, nutrients and drugs around the various compartments of the body, pathogenic (disease-causing) microbes are also carried in the blood of an infected person. The most notorious contemporary instance of this is, of course, the presence of the AIDS virus, HIV (human immunodeficiency virus) in the blood due to blood-to-body fluid contact with an infected person or syringe *etc*. Other notable blood borne diseases include malaria, caused by the protozoan parasite class *Plasmodium* spp., and there are annual outcries in the British winter due to meningitis and septicaemia, both caused by bacterial colonisation (*e.g.* by *Staphylococcus aureus*) of the bloodstream.

What happens if someone whose blood is contaminated donates? Hopefully they will realise they are ill and won't attend the local blood donors' session. Blood taken from those who feel well but are showing no symptoms (asymptomatic) and don't belong to the increasing list of 'at risk' classes in the donors' questionnaire will be routinely screened for pathogens using modern biochemical techniques.

Blood donations which fail the screening process are rejected, but there are times when the donor may have been infected only recently and the concentration of pathogens in the blood is too low to be detected at screening. On such occasions there is the potential for transfusion-transmission of infection. Obviously from the ethical as well as the human point of view this is most undesirable. Additionally (and increasingly) there is the consequence of litigation from the infected recipient. Blood is now a big business run mainly by multinationals, so not all product considerations are governed by beneficence. However, the requirement for clean blood derivatives is paramount from all standpoints. Additionally there is always the threat of the 'emerging pathogen', *i.e.* a previously unknown disease, for which by definition there is no screening test available. The AIDS virus was such a pathogen in the late 1970s and early 80s, with tragic consequences to many people with blood clotting disorders (*e.g.* haemophiliacs), who used infected products.

Mark Wainwright has worked in the area of photosensitizer research since 1987, and on light-activated antimicrobials for the past eight years. His main interest lies in the application of photosensitizers to pathogen inactivation, mainly against drug-resistant targets and in blood products. He is currently Reader in Medicinal Chemistry at UCLan.

 DOI: 10.1039/b101905h

As with standard antimicrobials, strategies for the disinfection of donated blood, should be based on selective medicinal chemistry. This requires definition of both the host and target cells.

1.2 Blood and blood products

When a blood donor gives his or her half litre, what results is a plastic bag and several sample ampoules of whole blood, pre-typed with a brief medical history. This whole blood may be used in direct replacement for blood loss in trauma or surgery, or it may be separated physically *e.g.* by centrifugation and temperature precipitation (cryoprecipitation) into a number of useful blood products.

Plasma is the aqueous fraction of the blood and contains the proteins and factors necessary for function—*e.g.* serum lipoproteins and clotting factors. In order to gain sufficient levels of these factors, it is necessary to combine several plasma fractions ('pooling') and this has ramifications in disease control. Plasma is often frozen promptly and thawed before use. Fresh frozen plasma (FFP) stored at −30 °C is allowed a shelf life of twelve months in the UK.

Platelet (thrombocyte) concentrates (PC) are required in the treatment of bleeding disorders. They are concentrated from plasma and may be stored at 22 °C for up to five days. Platelets are concentrated from multiple donations—concentration from at least four units (0.5 l) of blood is required to cause a significantly elevated level in the recipient.

Red blood cell concentrates (RBCCs, erythrocytes) are used in the improvement of oxygen-carrying capability due to blood loss either after trauma or surgery. Red blood cell concentrates can be stored at 4–6 °C for up to 35 days.

White blood cells (WBCs, leukocytes) are heavily involved in defence against disease. While they are vital in the bloodstream, in blood for transfusion they can cause pathogenic disease since they may be associated with the infectious agents. White cell reduction (leukodepletion) is therefore an important process.

The major difference between the fractions is cellular in nature. Plasma is an aqueous suspension of proteins and infecting microorganisms are also in suspension, whereas platelets, erythrocytes and leukocytes are different cell types and the pathogens may be intra-, extracellular or both. A further difference lies in the fact that WBCs contain nucleic acid where the other cell types, and plasma, do not.

2 Blood disinfection

Potentially, contaminating pathogens in blood cover a huge range. Colonisation by each of viruses, bacteria, yeasts and protozoa is possible, depending on the history of the donor, and the degree of care taken in the handling of the donation in the processing stage.

Historically, *i.e.* over the past 40 years, disinfection of blood has not been approached generally with the same zeal or funding as has mainstream antimicrobial development. Typically, treatment of blood fractions has been based on physical methods such as filtration, irradiation using X-rays or ultraviolet light, or chemical treatment based on solvents and surfactants. Of these, only the filtration systems offer any selectivity, and that being purely on size. Ionising radiation is not targeted and causes collateral damage to proteins and cellular material. Surfactants are toxic *e.g.* to some viruses because they disrupt the viral envelope, but the same thing occurs with blood cells.

Clearly a targeted approach to the removal or eradication of pathogens in donated blood would be preferable, both in terms of efficiency of cell kill and also—if a generic procedure could be developed for the treatment of all donated blood—in terms of cost.

The use of traditional antimicrobial agents here is discounted since what is required is activity against all pathogens (and probably white cells) rather than discrete antibacterial or antiviral activity. In addition, drug allergies would disbar the use of *e.g.* β-lactams in blood disinfection.

Since, in general, the requisite blood fractions (plasma, platelets and red blood cells) do not contain nucleic acids, but the infecting microorganisms and white blood cells do, the targeting strategy for most approaches to the problem have been aimed at denaturing or otherwise inactivating nucleic acid. As will be seen, this approach has taken several different paths—and immense amounts of funding—to a similar end: Pathogen Inactivation/Eradication Technology (PIT/PET) has become a firmly established aim for most of the 'blood industrials' and blood service providers.

Another obstacle is that of possible host toxicity to the recipients of treated blood products. This means ensuring that any agent used in PIT must be present post-treatment at negligible—and non-harmful—levels or not at all. The latter is preferable, since human cells generally contain nucleic acid, and the threat of possible mutagenicity, however miniscule, in the recipient should be avoided.

As stated above, blood products now represent a considerable global market and advances in the chemistry behind disinfective processes are often hidden in the patent literature. Such processes have been developed over the past fifteen years, mainly in industry, and can be categorised as either chemotherapeutic, photochemotherapeutic or photodynamic. The present review represents an attempt to discuss these various processes from an organic medicinal chemistry viewpoint.

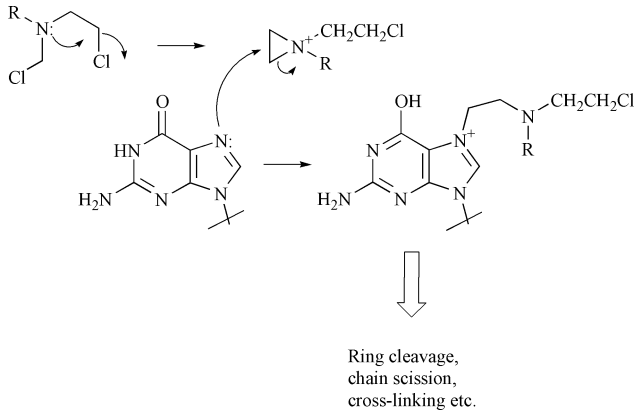
3 The chemotherapeutic approach—aziridine and pro-aziridine chemistry

During World War I the consequences of exposure to mustard gas—bis(2-chloroethyl)sulfide—were quite often tragic and disastrous. However, this simple molecule became the lead compound for cancer chemotherapy in the interwar years, with clinical trials of nitrogen mustards such as mechlorethamine (*N*,*N*-bis(2-chloroethyl)methylamine) in 1942. The effects of this agent on DNA became apparent soon after and this is still considered to be the major site of action of this class of agents.

The chemistry underlying mustard action is quite simple: the presence of the chloroethyl moiety on nitrogen forms an inherently electrophilic site. This is made more electrophilic *via* the formation of an aziridinium ring, the halogen being displaced intramolecularly by the nitrogen lone pair (Scheme 1). Nucleophilic sites in DNA, typically N-7 of guanine, attack the electrophilic carbon adjacent to the aziridinium nitrogen and this causes alkylation of the nucleic acid and subsequent degradative processes such as ring cleavage or DNA scission. Further alkylation may result in cross-linking of the double strand (Scheme 1).

The targeting of DNA in human tumours is notoriously difficult, the lack of selectivity of the early mustard-based drugs causing considerable side effects and toxicity. This is also a manifestation of their high reactivity—there are many more cellular nucleophilic sites available outwith DNA.

In order to overcome the targeting problem, use was made of chromophores which were known to be selective for nucleic acid. One such is the acridine chromophore, antibacterial drugs containing which were shown to interact with DNA by stacking (intercalating) between the base pairs. The antimalarial drug mepacrine (Fig. 1) contains both an aminoacridine nucleus and a pendant diethylamino group suitable for replacement with the



Scheme 1 Aziridinium ion formation by a nitrogen mustard and nucleic acid reactions.

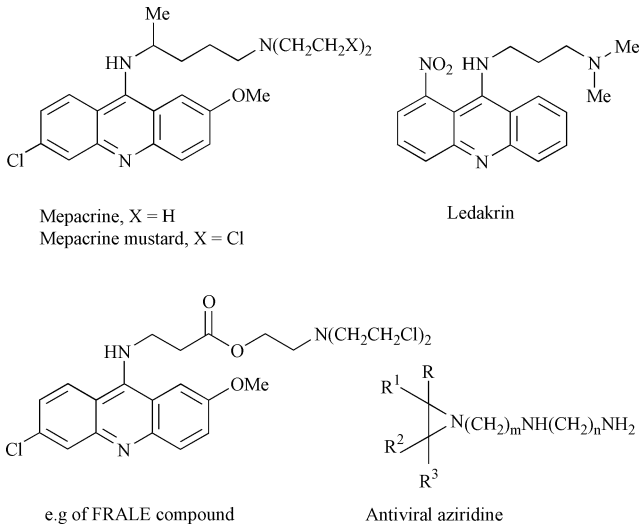


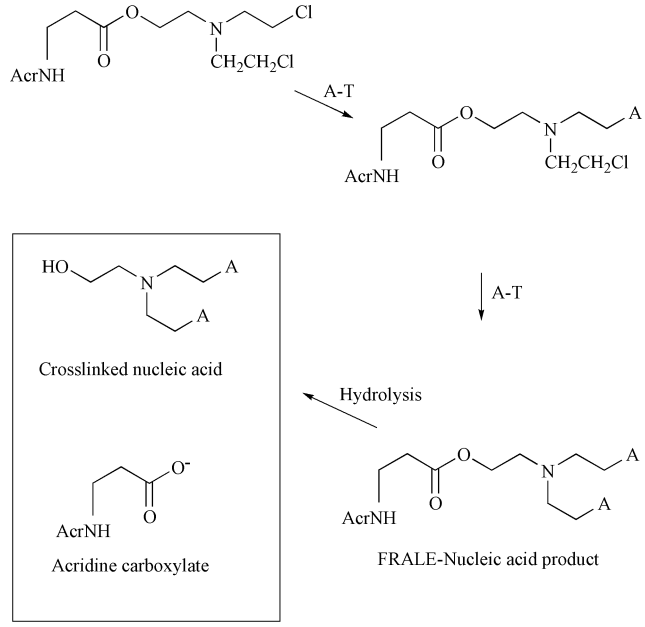
Fig. 1 Acridine intercalators: mepacrine, mepacrine mustard, Ledakrin and FRALE structure. Generalised antiviral aziridine structure, R groups = H, alkyl.

nitrogen mustard moiety, allowing the use of mepacrine mustard (Fig. 1) in chemotherapy. The acridine chromophore effectively carries the reactive group to the DNA, and this has been shown with other anticancer agents such as the nitroacridine derivative Ledakrin (Fig. 1).

DNA intercalation by the acridine nucleus depends on both its size and shape, and the fact that it is cationically ionised at physiological pH when suitably substituted. This usually requires a fully conjugated amino substituent (*i.e.* occupying position-3, -6 or -9 of the ring). Anionic acridines (*e.g.* acridinecarboxylic acids) do not intercalate.

The use of mustard/aziridine methodology in blood product disinfection is simpler in practice than cancer chemotherapy. Mainly aimed at the decontamination of red blood cell concentrates, the Cerus Corporation has patented an ingenious acridine-containing mustard with a difference.[2] Such FRALE (Frangible Anchor Linker Effector) compounds utilise the intercalating activity of the acridine nucleus as above in conjunction with mustard alkylation of nucleic acid, but the two moieties are connected by an ester linkage which is easily hydrolysed post-alkylation (thus 'frangible', Scheme 2). The resulting acridine is a carboxylate which does not intercalate and so poses no threat in terms of recipient mutagenicity. Targeting nucleic acid *e.g.* in viral contaminants is possible whether the pathogen is intra- or extracellular, since mepacrine analogues can cross into the interior of erythrocytes (thus their use as antimalarials).

Simpler aziridines (ethylenimines) are also proposed for the decontamination of red blood cell concentrates. Work by Vitex



Scheme 2 Mechanism of FRALE action, A–T = adenine–thymine base pair.

and associated companies has resulted in *N*-alkylaminoalkyl substituted aziridines[3] (Fig. 1) which are reportedly nucleic acid specific, binding *via* ionic interactions between ionised (protonated) amino groups in the side chain and negatively charged phosphates in the nucleic acid backbone allowing proximity and consequent reaction between the requisite electrophilic (aziridinium) and nucleophilic groups. Nucleic acid alkylation and its sequelae are thus facilitated similarly to the FRALE mode of action described above, the main differences lying in the targeting strategy and the fact that the reactive moiety is present in the starting material and activated by protonation.

4 Light activated chemistries

Both the photochemotherapeutic and photodynamic approaches to blood disinfection employ light as a source of energy for chemical reactions at the target site. The reactions can be classified broadly as either cycloadditive or photooxidative respectively, although there is a degree of commonality in the two methods. Since both employ light energy for reaction it is necessary to give an overview of the mechanism and consequences of photoactivation (Fig. 2).

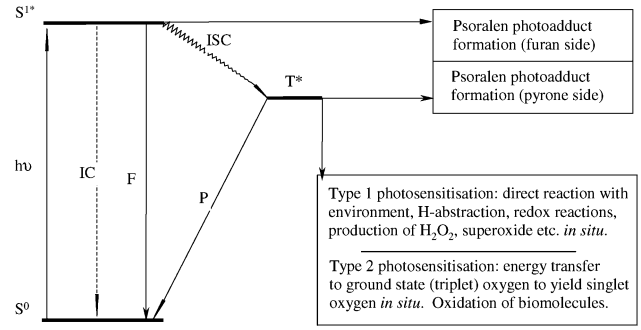


Fig. 2 Photochemical / photosensitization pathways. Key: $h\upsilon$–light absorption; IC–internal energy conversion; F–fluorescence; P–phosphorescence; ISC–intersystem crossing.

In most cases, light absorption by organic compounds relies on the presence of a π-system (chromophore). Thus, simple

alkenes and carbonyl species as well as more complex aromatic dyes all have the potential for photochemical reaction utilising π-electrons or those associated with heteroatomic lone pairs (n). Generally, the realisation of useful therapeutic photochemical potential relies on the absorption of light of the correct wavelength to promote an electron from the singlet electronic ground state (S^0, electron spins paired) to the excited singlet state (S^{1*}) of the chromophore ($\pi \rightarrow \pi^*$ or $n \rightarrow \pi^*$). Significant and long-lived population of the singlet excited state of the chromophore facilitates photochemical reaction—and the stability of the excited state may be increased by the inclusion in the chromophore of an atom of higher atomic mass (the 'heavy atom effect')—while pathways such as fluorescence allow deactivation to S^0. Efficient intersystem crossing to the excited triplet state (T*, electron spins unpaired) is also required. Both photochemical and photosensitizing reactions are possible from the T* state (Fig. 2).

For photochemotherapeutic reactions, *i.e.* those typically involving psoralen-type molecules and UVA (300–400 nm), different photochemical products occur depending on whether the reaction occurs at the singlet or triplet stage.[4] Photodynamic action, classified as Type I (redox) or Type II (singlet oxygen-mediated) occurs from the triplet excited state of the photosensitizer (Fig. 2).

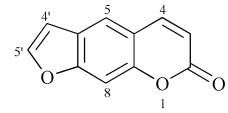
Regardless of the type of activating energy, it should be remembered that the practical aspect of pathogen inactivation in blood products requires the treatment of large volumes of liquid, usually contained in plastic bags of the order of hundreds of millilitres. Consequently, the use of pinpoint accuracy in light delivery, as in the related photodynamic treatment of tumours is of little use. Rather a 'light table' or other illuminating surface must be employed, supplying a large surface area of low power (milliwatt) white or fluorescent light on which a number of bags can be laid for treatment.

A further complication lies in the presence in red blood concentrates of haem pigments (in haemoglobin). Platelets and plasma are substantially transparent above 300 nm, but haemoglobin has several absorption bands in the range 300–630 nm. Therefore photoactivated compounds for use in RBCCs should exhibit significant absorption outside this spectrum (*i.e.* at longer wavelengths) and for this reason psoralens are of little utility in RBCC treatment. With the exception of riboflavin, photosensitizing compounds employed here have generally been based on chromophores with absorption bands above 600 nm, for example the phthalocyanines and phenothiazinium dyes.

4.1 Photochemotherapeutic agents—psoralens and photoadducts

The use of psoralens (furocoumarins) in the phototherapy of psoriasis (PUVA) is well established. The related treatment of lymphoma utilises removal of blood and photochemical treatment of the aberrant white cells after separation from other constituents.[5] The parallel with blood disinfection is obvious and psoralen research in this area probably represents the major contribution in light activated methodologies.

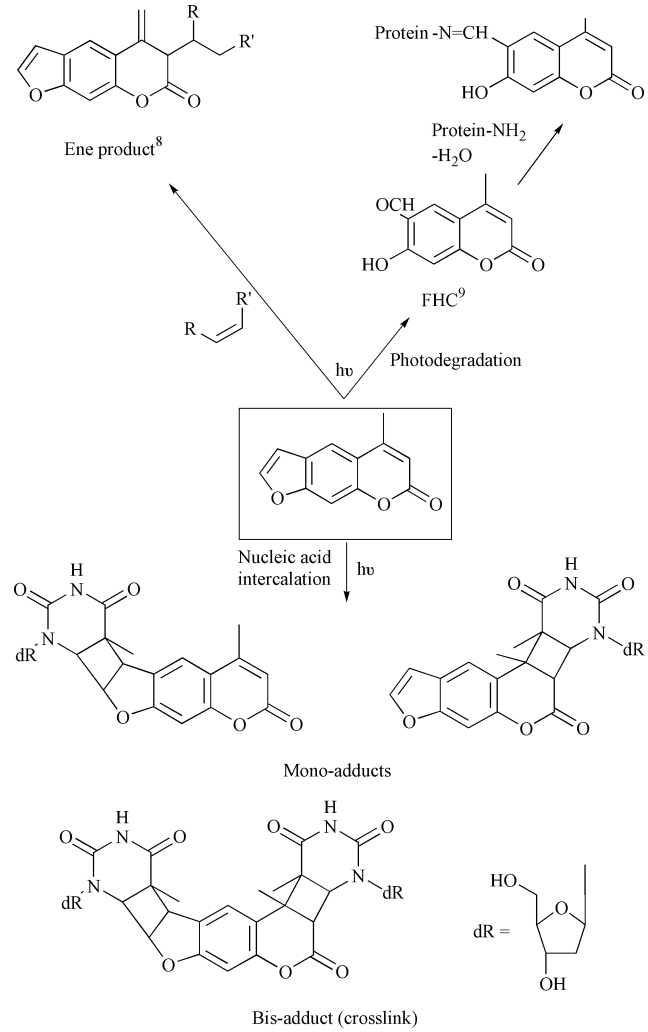
Like the acridines, psoralens are effective nucleic acid intercalators—indeed the patent covering FRALE compounds referred to above also cites psoralen chromophores as possible 'anchors'. Linear furocoumarins (Fig. 3) absorb long-wavelength ultraviolet light, usually in the region 300–340 nm (*e.g.* 8-methoxypsoralen λ_{max} 300 nm, 4,5′,8-trimethylpsoralen λ_{max} 335 nm, Fig. 3), wavelengths between 300–400 nm being employed routinely to activate the psoralen compounds in practice. Absorption of UV light by the nucleic acid target occurs at lower wavelengths (λ_{max} *ca.* 290 nm) and so it is little affected by the light source.



	R^4	$R^{4'}$	$R^{4'}$	R^8
8-MOP	H	H	H	MeO
4,5′8-TMP	Me	H	Me	Me
AMT	Me	H_2NCH_2	Me	Me

Fig. 3 Furocoumarins.

For the purposes of photochemotherapy, intercalated psoralens may thus be excited *in situ*, causing the promotion of [2 + 2] cycloaddition reactions with olefinic moieties in nucleotide bases, such as cytosine (Scheme 3). The formation of mono- or



Scheme 3 Psoralen and photoproduct modes of action.

bis-adducts is possible, either of which damages the nucleic acid, causing cross-linking in the latter case, although it is established that mono-adducts from the furan side of the molecule result exclusively from the singlet excited state, pyrone-adducts mainly from the triplet (Fig. 2).[6] Psoralen-type compounds also have the ability to produce singlet oxygen on illumination and photooxidative damage at the site of action may also occur (see Photosensitizer section).

Although the typical biological photoreaction of psoralens has been established as [2 + 2] addition with nucleic acid, the furocoumarin nucleus will undergo this reaction with other olefinic moieties, for example in unsaturated fatty acids.[7] The importance of nucleic acid targeting by the psoralen can thus be seen in terms of possible side-reactions, *e.g.* at the platelet membrane. Such reaction would leave the membrane altered

chemically, allowing potential immune response in the recipient as reported in the related PUVA therapy.[5] The ene reaction has also been reported between photoexcited psoralens and olefins (Scheme 3).[8]

Other side reactions which might be of greater significance involve the formation of labile psoralen breakdown photoproducts such as 6-formyl-7-hydroxycoumarin (FHC, Scheme 3).[9] Aldehyde-containing photoproducts of this type form Schiff's bases with amine-containing molecules, thus protein adducts are possible, *e.g.* again at membrane surfaces (Scheme 3). This may also provide a route to reported DNA–protein cross-linking as a result of psoralen phototreatment.

Evolving from the use of psoralen natural products in phototherapy, the application of the series to blood product decontamination began with 8-methoxypsoralen (8-MOP) as a lead compound. However, low aqueous solubility meant that new compounds were required for the efficient application of psoralen photochemistry to blood products. Among a range of novel compounds synthesised, 4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT, Fig. 3) satisfied this criterion, the amino moiety being protonated in the physiological pH range. The cationic nature of AMT also promotes nucleic acid binding compared to 8-MOP, being approximately tenfold higher for the daughter compound. However, this led to concerns over its potential for increased mutagenicity.[10] However, in terms of blood product disinfection, AMT proved to be an excellent lead candidate for plasma and platelet fractions, exhibiting high activity across the range of pathogenic types.

Such positive evidence of activity in plasma and platelet fractions promoted considerable proprietary research activity aimed at developing a commercial product based on AMT, with lowered mutagenic potential. The Cerus/Baxter psoralen preparation S-59 appears to be a considerable advance in this area.[11] Although the structure of this psoralen has not been specified, it is described as an aminoalkylated psoralen, having structural features in common with both 8-MOP and AMT. S-59 has exhibited high levels of activity against viruses (including HIV and cytomegalovirus) and bacteria, and in leucoreduction. S-59 has been undergoing clinical trials since 1997.

The recent improvements in psoralens offer an effective approach to pathogen inactivation in plasma and platelet fractions. However, since red blood cells contain significant levels of haem pigments, the short wavelengths of light required to activate the intercalated psoralen molecules and produce photoadducts are absorbed, and therefore effectively screened, by the haem content of the cell. For this reason, photoinactivation techniques for use in RBCCs require photosensitizers having longer (visible) absorption wavelengths, *i.e.* outwith that of haem.

4.2 Photodynamic agents—natural and synthetic photosensitizers

The photodynamic therapy of cancer (PDT) uses a combination of a photosensitizing drug and light to give rise to reactive oxygen species in the tumour environment, leading to tumour death. As an antimicrobial approach, the use of this technique predates PDT but remained relatively unexplored due to the ease of access to antibiotics. However, in the age of drug-resistant bacteria, AIDS and consequent generally increased viral awareness, photodynamic antimicrobial chemotherapy (PACT[12]) has a potential niche market in local decontamination, including its use in blood products. The putative employment of PACT in the wound clinic may be limited to localised infection due to the problems of systemic light delivery, but the disinfection of blood products is, by definition, a localised treatment, and the illumination of a blood bag is straightforward.

However, local treatment in terms of the addition of a photosensitizer and subsequent illumination of a blood bag still entails treatment of the whole volume and might engender collateral damage to blood constituents (proteins *etc.*) or to toxicity in subsequent transfusion recipients. Thus—as with standard chemotherapy—photokilling protocols should be carried out at the lowest photosensitizer concentration possible, and the quantity and quality of light used is also important. The power density or fluence rate of a light source is normally given in mW cm^{-2} whereas the light dose or fluence gives the energy received (*e.g.* by a blood bag), calculated as a product of the power density and the illumination time (in seconds). The power density or the illumination time can therefore be varied for the same light dose (J cm^{-2}). However, for a given photosensitizer concentration, a high power density over a short time period may give a different rate of microbial kill—and collateral damage—to that of a low power density over a longer time, although the light dose is the same in each case.

Photodynamic microbial damage at the molecular level is well established. Type I photodamage (Fig. 2) due to hydrogen or electron abstraction by the photosensitizer, subsequently initiated redox reactions and oxygenation products relies on the photosensitizer being in close proximity to its biomolecular target. Type I reaction with oxygen in the microbial environment (or cytoplasm) can give rise to superoxide and thus hydroxyl radicals (HO·) which will undergo further reaction with biomolecules or combine to give hydrogen peroxide *in situ*, again leading to cytotoxic events.

Type I reactions (Fig. 2) include the abstraction of allylic hydrogens from unsaturated molecules such as phospholipids, *e.g.* in the bacterial plasma membrane. Reaction of the radical species thus formed with *in situ* oxygen leads to lipid hydroperoxide formation. Lipid peroxidation has detrimental effects on membrane integrity, leading to a loss of fluidity and increased ion permeability. For example, aminolipids and peptides in the viral envelope are potential targets, leading to the inactivation of membrane enzymes and receptors.[13]

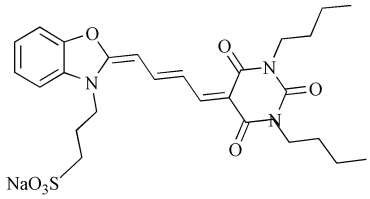
Type II processes (Fig. 2) are generally accepted as the major pathways in photooxidative microbial cell damage in blood phototreatment, due to the ready availability of molecular oxygen. As with the Type I pathway discussed above, singlet oxygen will also react with molecules involved in the maintenance and structure of the cell wall/membrane such as peptides, phospholipids and sterols. Reactions of singlet oxygen with other molecules involved in the cell wall/membrane can also occur. These may be straightforward, such as S-oxidation (methionine to methionine sulfoxide), or cycloadditive, such as the reaction with tryptophan residues, the unstable intermediate product formed degrading to give *e.g.* aldehyde derivatives which may result in peptide crosslinking.[14]

It is established that nucleic acids react to photodynamic treatment mainly through guanosine residues.[15] Again there exists a difference in selectivity between Types I and II processes. The former is mediated through hydroxyl radical attack at the sugar moiety whereas the latter involves attack of singlet oxygen at the guanine base.

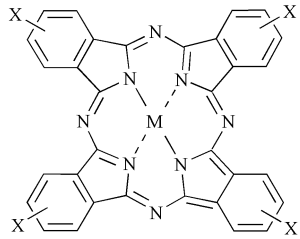
The distribution between pharmacological compartments and the site of action of photodynamic agents is, of course, governed by the standard steric and physicochemical principles familiar to the mainstream medicinal chemist—lipophilic character, degree and type of ionisation, structural planarity *etc.* Thus, although the focus of antiviral activity of photosensitizers has traditionally been reaction with nucleic acid, the variety of photosensitizer structures employed must logically contradict this.

4.2.1 Cyanines. Given the range of cyanine dyes used in photographic sensitisation, it is surprising that the combination of visible light absorption and previously proven antimicrobial

activity has not encouraged a great deal more original research in this area. As part of the research effort in photodynamic therapy, Merocyanine 540 (MC540, Fig. 4) has been used in the



MC 540



Metallophthalocyanine derivative

Fig. 4 MC540 and phthalocyanines.

purging of leukaemic cells from autologous bone marrow grafts, and this led to its investigation in the photoinactivation of blood-borne enveloped viruses.[16]

The chemical structure of MC540 is appealing to the medicinal chemist as it offers much scope for functionalisation. Thus, ring heteroatom exchange for one of higher atomic number (*e.g.* oxygen for sulfur) normally leads to increased singlet oxygen yields[17]—the 'heavy atom effect' (*vide supra*)—though for cyanine dyes these are usually low in comparison to *e.g.* methylene blue. The use of heavy atoms also stabilises the polymethine chain to photoisomerisation (a major deactivation pathway).[17] As expected, varying the *N*-alkyl side chains in MC540 facilitates changes in the lipophilicity of the system. Perhaps the main drawback to the use of merocyanines is the inactivation of such compounds by plasma, *e.g.* in the attempted photoinactivation of herpes simplex-1 virus, although recent work has shown that such effects may be avoided by the replacement of the ring oxygen with sulfur or selenium.[18] MC540 is thought to exert its effects at the viral envelope. However, photodamage to the erythrocytic membrane has also been reported.[19]

4.2.2 Phthalocyanines. As noted previously, light absorption by the haem of RBCCs is a determining factor in the design and development of phototreatments for this blood fraction. This partly explains why haematoporphyrin derivative (HpD), widely used in cancer PDT, can not be employed in this instance. Another reason is the poor performance of anionic photosensitizers against microbial species. Although the phthalocyanines may be considered to be porphyrin derivatives (Fig. 4), greatly increased aromatic character explains the more intense near infrared absorption of these compounds compared to that of the parent porphyrin nucleus. In addition, straightforward synthetic routes to the phthalocyanines furnish a wide range of compounds in terms of the central metal/semi-metallic atom (*e.g.* M = Al, Ga, Si, Zn, Fig. 4) and side chain type. As photosensitizers, phthalocyanines are generally efficient in the production of singlet oxygen—offering improvements on that of standard photosensitizers such as methylene blue. However, as with the porphyrins, there is little evidence of a historic use of phthalocyanines either in the treatment or staining of microbes, and the considerable current interest in this class of photosensitizers should be seen as a development of cancer PDT research.

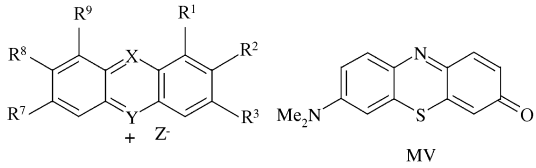
Phthalocyanines have shown significant potential in the area of blood product disinfection[20] although strangely, unlike methylene blue, phthalocyanine products have not yet reached the market-place, and phthalocyanine research in this area was recently discontinued by the Vitex Corporation. However, the efficacy of phthalocyanines in the photoinactivation of viruses in various components is considerable. Here, enveloped viruses such as HIV, herpes simplex (HSV) are generally amenable to photoinactivation whereas, for example, the non-enveloped cephalomyocarditis virus is not, indicating that the viral envelope may be a target for phthalocyanine photosensitization (*i.e.* rather than nucleic acid). The use of phthalocyanines against various forms of HIV infection has been reported, and once again paralleling the development of these compounds in cancer PDT, aluminium and silicon phthalocyanines appear to show considerable promise, both groups of compounds exhibiting light absorption in the far red, along with high efficiencies of singlet oxygen production.[20] The silicon phthalocyanines used as photodynamic agents are functionalised axially through the silicon atom, rather than in the periphery of the ring and this appears to produce highly active compounds. Thus, for example, silicon phthalocyanine (Pc4) bearing a cationic dialkylaminoalkylsilyloxy-residue on the central silicon (Fig. 4, X = H, M = $Si(OH)OSiCH_2CH_2NMe_2$) was not only active against cell-free HIV but also against the actively-replicating virus and latently infected red blood cells (using a photosensitizer concentration of 2 μM and a power density of 25 mW cm^{-2}).[21] Such an activity profile against viruses is obviously highly desirable although, as with much of the phthalocyanine work, red blood cell damage was apparent which required the addition of an antioxidant such as vitamin E.[22]

Logically, in terms of structure–activity relationships for the phthalocyanines, there appears little general correlation between the antiviral potency and the central atom of the phthalocyanine, since this has more effect on singlet oxygen yield than selectivity and binding. However, the degree of phthalocyanine butylation and sulfonation (Fig. 4, X = Me_3C– or SO_3H)—both of which have a more direct effect on microbial uptake and localisation—affected both the antiviral activity and also the extent of haemolysis.

Although widely publicised, viral contamination is not, of course, the sole factor in disease transmission through donated blood.[12] It has been reported that blood-borne pathogens involved in tropical diseases may be inactivated using PACT. Thus the cationic silicon phthalocyanine mentioned above has been shown to mediate the photoinactivation of parasites such as *Plasmodium falciparum* and *Trypanosoma cruzi* under similar conditions to those stated above (2 μM photosensitizer/25 mW cm^{-2} illumination). Indeed, the Pc4 compound mentioned above was such an effective antiplasmodial that it has been used as a lead compound in antimalarial research.[23]

Photobactericidal testing of phthalocyanines has also been carried out, *in vitro*. Thus Wilson *et al.* have demonstrated the photokilling of a range of bacteria in biofilms and also of methicillin-resistant *Staphylococcus aureus* using aluminium phthalocyanine.[24] Testing of anionic, cationic and neutral zinc phthalocyanines against both Gram positive and Gram negative bacteria showed that only the positively charged phthalocyanine (a pyridinium salt: X = $–CH_2N^+C_5H_5$, Fig. 4) was active.[25] This indicates the presence of a specific site of action for the active species since the neutral phthalocyanine showed similar uptake without activity. Unfortunately, little work has been reported thus far on the photobactericidal effects of these compounds in the presence of blood components.

4.2.3 Phenothiazinium photosensitizers. Methylene blue (MB, Fig. 5) is an established biological stain and an effective photosensitizer. In terms of blood disinfection it satisfies most



MV

	X	Y	R^1	R^2	R^3	R^7	R^8	R^9
MB	N	S	H	H	NMe_2	NMe_2	H	H
AO	CH	N	H	H	NMe_2	NMe_2	H	H
DMMB	N	S	Me	H	NMe_2	NMe_2	H	Me
NMB	N	S	H	Me	NHEt	NHEt	Me	H
TBO	N	S	H	Me	NH_2	NMe_2	H	H
AA	N	S	H	H	NH_2	NMe_2	H	H
AB	N	S	H	H	NHMe	NMe_2	H	H
AC	N	S	H	H	NH_2	NHMe	H	H
Th	N	S	H	H	NH_2	NH_2	H	H
MVOMe	N	S	H	H	OMe	NMe_2	H	H

Fig. 5 Phenothiazine-derived photosensitizers and acridine orange.

of the requisite performance criteria, since it is also an efficient nucleic acid intercalator, is demonstrably non-toxic in humans and exhibits intense absorption at 660 nm. In addition, the chemical synthesis of phenothiazinium compounds is well understood, allowing facile derivative preparation, normally *via* oxidative cyclisations of *p*-phenylenediaminethiosulfonic acids and aniline derivatives, or the oxidative amination of 10*H*-phenothiazine. Although work in this area is thus broadly based upon the character of the substituted amino group in the aniline starting material, only scant research has appeared in the literature concerning the inclusion and effects of different atoms or groups in the periphery of the phenothiazinium ring system.[26]

Although methylene blue is an effective intercalator, due to the positive charge and sufficient planar surface area of the photosensitizer, the chromophore is reduced in biological systems to the neutral 10*H*-phenothiazine species and the antibacterial activity of methylene blue is correspondingly low. Acridine orange (AO, Fig. 5) is bioisosteric with methylene blue, differing only in the heteroatomic character of the central ring, yet the former exhibits significantly higher antibacterial activity since it is less easy to reduce and so maintains a high pK_a in the biological milieu, the cation facilitating DNA intercalation. It has been reported recently that chromophoric methylation of methylene blue decreases the tendency to reduction and that 1,9-dimethyl methylene blue (DMMB, Fig. 5) is considerably more bactericidal *in vitro* than either methylene blue or acridine orange.[27]

While MB derivatives are encountered as cations, deprotonation is possible, in the presence of a conjugated primary or secondary amino group at position-3 or -7 of the ring system, furnishing a neutral quinoneimine. Phenothiazinium salts such as new methylene blue (NMB), toluidine blue O (TBO) and the azure stains (AA, AB, AC Fig. 5) will behave thus. Such cation-neutral facility is likely to be important in cellular uptake. Neutral species are typically much more hydrophobic than their ionic counterparts, and as such, the quinoneimines should exhibit improved partitioning behaviour *e.g.* at membrane surfaces. This is important from the viewpoint of uptake into cellular blood components, and thus allowing intracellular activity. Methylene violet (MV, Fig. 5) is produced by the hydrolysis of MB and thus has an oxo-function at position-3 in place of *N,N*-dimethylamino. It is thus also normally encountered as the neutral species.

Whereas methylene blue is hydrophilic, the lipophilicity of MB derivatives may also be increased by standard functionalisation. For example dimethylation in the ring (furnishing DMMB, Fig. 5) allows partitioning into ether, octanol *etc*. This increased hydrophobicity in conjunction with a permanent positive charge is a factor in the improved antimicrobial profile of dimethyl methylene blue compared to the parent compound.[27]

Phenothiazinium dyes such as methylene blue and toluidine blue O are well established as systemic/vital stains which are used routinely during surgical procedures—for example, the staining of the oral mucosa is normally carried out at millimolar levels. Post-transfusion toxicity from, *e.g.*, treated plasma products where disinfection requires micromolar amounts of the photosensitizer is thus unlikely for the phenothiazinium photosensitizers, although the Paul Ehrlich Institute recently refused to license methylene blue-treated plasma. While the toxicities of other phenothiazinium derivatives are less well proven, the principle is established at least for the lead compounds. In addition, a range of phenothiazinium dyes has been employed over the past *ca*. 80 years in the staining of blood samples. Indeed, viral photoinactivation using the standard phenothiazinium stains such as methylene blue and toluidine blue O has been known since 1928.[28]

Although the planar structure and positive formal charge of the phenothiazinium salts underlines the likely mode of action as photodamage to nucleic acid, as mentioned above the actual target sites in viruses depend on several factors, including the derivative used as well as virus type.[29] This is unsurprising given the variety of structures within the series (Fig. 5). Although viral nucleic acid is a significant target for photodamage using MB, this may occur in various ways including base modification rather than direct strand breakage, and the formation of nucleic acid–protein crosslinks. Other sites of damage include internal viral protein or lipid, viral envelope and viral core proteins, viral ribonucleic acid and reverse transcriptase. Both toluidine blue O and methylene violet are known to cause nucleic acid photodamage, although the latter acts in part *via*direct electron transfer to the nucleotide bases (causing 'nicks') and also by photoadduct formation.[30] The latter mechanism is not seen with the phenothiazinium cations, although a mixture of Type I and Type II mechanisms depending on the oxygen tension has been reported. As has been mentioned, it is important that both enveloped and non-enveloped viruses are susceptible to the photodynamic agent used. While it is apparent that MB is less effective against the latter type, both MB and TBO have been shown to inactivate adenoviruses *via*nucleic acid damage. Doubts concerning the efficacy of MB against some non-enveloped viruses may be remedied by changing the photosensitizer structure, since each of the demethylated methylene blue derivatives (azures A,B,C and thionin, Fig. 5) has been found to be suitable for virus inactivation in plasma. In addition, thionin has reportedly improved photoantiviral activity in platelets, compared to MB, with lower collateral damage. The performance of thionin (λ_{max} 598 nm) in red blood cells may be lowered due to endogenous light absorption, and its efficacy in plasma-containing fractions is also lower.

Methylene blue has been widely used by several European transfusion services in the photodecontamination of blood plasma and has been shown to be particularly effective in the inactivation of enveloped viruses. Both the commercial *Pathinact-MB* (Baxter) and *Blueflex* (MacoPharma) systems employ this photosensitizer. The adoption of the MB-mediated technique was first suggested by research carried out at the Walter Reed Institute in 1955 but was not used routinely by blood services until 1992 (in Europe).[31,32]

To allow intracellular activity and decrease collateral photodamage, the drive for improved MB derivatives has been based on increased lipophilicity. In this respect, particularly due to excellent work by Wagner *et al*., the main contenders to emerge are methylene violet and 1,9-dimethyl methylene blue. As already mentioned, methylene violet is encountered as a neutral quinonoid species (Fig. 5) and while this ensures intracellular localisation, and reasonable activity against intracellular viruses is also reported to lead to plasma binding which in fact decreases the potential activity.[33] Positively-charged methylene violet derivatives, *e.g.* produced by virtue of *O*-methylation of

the parent, may address this shortfall, although the *O*-methylated derivative (MVOMe, Fig. 5) exhibited a much decreased singlet oxygen yield (0.054 compared to 0.32).[34]

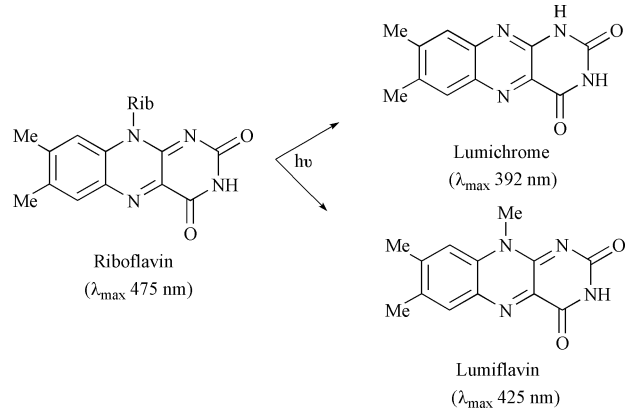
Dimethyl methylene blue (DMMB) is a commercially available stain ('Taylor's Blue') which owes its increased lipophilicity to increased alkylation of the MB structure. Compared to the parent compound—and also to more lipophilic analogues[35]—DMMB exhibits improved singlet oxygen yield, lower reduction potential[36] and higher affinity for nucleic acids.[35] In terms of pathogen inactivation, the dimethyl derivative represents a great advance, being highly efficient against both extra- and intracellular viruses and non-enveloped species. Additionally, the nucleic acid binding activity is important in the use of this compound for leucodepletion. DMMB is also a highly efficient photobactericide against a range of bacteria, again offering much improved performance relative to MB.[27] The intermediacy of singlet oxygen (*i.e.* Type II photosensitization) in DMMB virus-inactivation has recently been confirmed as the major mode of action, although the possibility of a Type I mechanism for the production of DNA nicks, particularly in the presence of water, has also been suggested.[37] The main problem in the use of the dimethylated derivative appears to be that of potential toxicity to the recipient. This has not yet been reported fully, although in anticancer testing in culture by the author, inherent (dark) toxicity appeared to be high for DMMB itself, at micromolar levels, compared to that of methylene blue.[36] However, in line with MB itself, work has been carried out on photosensitizer removal post-treatment, *e.g. via* filtration.

New structures based on the phenothiazinium chromophore are still required in order to address broad-spectrum antimicrobial activity and lower toxicity/collateral effects. The author has shown that new methylene blue (NMB, Fig. 5), which again has a dimethylated phenothiazinium structure, exhibits equal or greater photobactericidal efficacy compared to DMMB with lower toxicity in mammalian cell lines.[27] In addition, NMB was eight times as active as DMMB in a recent trial against *Yersinia enterocolitica*, a Gram-negative organism which is known to colonise red blood cell fractions during storage.[38]

Possibly because of the medicinal longevity of methylene blue and its congeners, and unlike the other chromophoric photosensitizer types, there is also considerable literature evidence of antifungal, antiplasmodial and trypanocidal activity in the phenothiazinium series. As MB is already marketed for blood disinfection this indicates a promising future for this class of photosensitizers.

4.2.4 Riboflavin. As the preceding review demonstrates, there are several routes available for the disinfection of blood products. Even within the sub-group of photodynamic agents currently under investigation, the number of possible agents is considerable, and the structural possibilities for future work enormous. However, outside antimicrobial, or photoantimicrobial, efficacy the main concern with treatments for blood disinfection lies in the likelihood of side effects or toxicities of the various agents in recipients, post-transfusion. This is currently requiring considerable research effort in the development of clean-up or removal technologies. One way to circumvent this is to use a proven, and accepted, non-toxic agent for the decontamination process.

Riboflavin, vitamin B2, is one such agent, being an essential dietary requirement in humans, rather than a xenobiotic. The photochemistry of riboflavin is well understood, including its breakdown to the useful secondary photosensitizers lumichrome and lumiflavin (Scheme 4), as are its interactions with nucleic acids. This, along with the demonstrable absence of toxic side effects, has led Goodrich and coworkers to use it in the current context.[39]



Scheme 4 Riboflavin and its photoproducts.

Although the work on riboflavin is at an extremely early stage compared to the other photosensitizers described, and as such there is scant contemporary literature available outside conference abstracts, riboflavin is claimed to be active in each of the major blood components without significant component damage. This includes its use in red blood cells, which is surprising in view of its short excitational wavelength—riboflavin has a maximum wavelength of absorption at 475 nm and another at 444 nm, *i.e.* at slightly longer wavelength than the Soret band of the haem pigment at *ca.* 410 nm. The introduction of riboflavin into red blood cell decontamination is likely to be a long-term goal.[39]

5 Conclusions

The use of selective methods of disinfection in the preparation of safe blood products has been introduced in various parts of the world over the past decade, but in a piecemeal manner. More reliable technologies and methods will enter the mainstream in the short term giving improved, more reliable blood products to those health concerns able to afford them. Among these, those protocols employing the psoralen S-59 (for plasma and platelets) and methylene blue (for plasma) appear to have been favourably received thus far. The main obstruction to be overcome is likely to be the ethical approval of DNA-targeting agents, or obtaining convincing proof that these can be completely removed before transfusion of the treated product.

While research into disinfection is well established, the application of the technologies discussed above to the removal of prion agents from donated blood is far more problematic. Prions are proteins and so must be targeted in a different way to nucleic acid. Workers are thus currently only in the initial stages of research into a targeting mechanism. Possible lead compounds in this area are Congo Red and its congeners,[40] being well known stains for amyloid, but recent work by Caughey *et al.* have shown that phthalocyanines and porphyrins are inhibitory also.[41] As the prion agent is likely to be carried in the plasma, pooled plasma is once again a risk (indeed native pooled plasma is not currently employed in the UK). Plainly this is an emergent problem which needs to be addressed quickly.

Synthetic blood/blood substitutes. The idea of a synthetic material as a replacement for blood has been under investigation for over fifty years, since it was shown that a mouse was able to continue breathing when immersed in a perfluorocarbon due to the inert nature and high oxygen solubility of the solvent. The area of blood substitutes is the subject of an excellent recent review.[42] A major problem to address lies in the physical properties of the materials as part of the circulatory system,

where surfactant adjuvants have been employed to improve flow.

As an alternative approach, cell-free haemoglobins (Hb) offer effective oxygen transport and exchange properties, similar to that of RBCs. Chemically such materials are of the cross-linked/polymerised Hb or polyethylene glycol–Hb conjugate type.[43] Current work is concerned with overcoming the potential for oxidative damage, *e.g.* by the use of polyhaemoglobin–superoxide dismutase–catalase.[44]

Blood substitutes are obviously attractive from the point of view of availability—polyhaemoglobin has a circulation half-life of 24 h, and can be stored for more than a year[44] (*cf.* 35 days for RBCs). They are also attractive to groups and religious sects averse to the use of human blood in operations (*e.g.* Jehovah's Witnesses).[45]

It should be noted that such replacement blood products represent oxygen carrier replacement rather than clotting factor preparations, and that there is evidence of an increased susceptibility to infection in the recipient.[46]

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