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Methylene Blue Based Protein Solder for Vascular Anastomoses: An In Vitro Burst Pressure Study

Jeremy F. Birch, FRCS,[1]* David J. Mandley, PhD,[2] Sian L. Williams, PhD,[2] David R. Worrall, PhD,[2] Patrick J. Trotter, PhD,[2] Francis Wilkinson, FRCS,[2] and Peter R. Bell, FRCS[1]

[1]Department of Surgery, RK CSB, Leicester University, Leicester LE7 5WW, United Kingdom
[2]Department of Chemistry, Loughborough University, Loughborough LE11 3TU, United Kingdom

Background and Objective: **Attempts at sutureless anastomoses have used protein-based solders containing chromophores [Oz et al., J Vasc Surg 1990;11:718; Poppas et al., J Urol 1998150:1052] to enhance the strength of laser anastomoses. Reports have described the use of indocyanine green [Oz et al., Surg Forum 1989;316.], fuschin, and fluorescein isothiocyanate as chromophores [Chuck et al., Lasers Surg Med 1989;9:471; Vance et al., Lasers Med Sci 1988;3:219]. Methylene blue (MB) is a chromophore with absorption peaks in the 600–700 nm region whose use has not been reported in laser-assisted vascular anastomoses. Therefore, we set out to produce and characterise a MB-containing protein solder. The absorption and burst pressure characteristics have been investigated and described as well as a brief review of the chemical and biological properties of MB.**

Study Design/Materials and Methods: **The MB and porcine serum albumin (PSA)–based solder was produced and used to form end-to-end anastomoses in porcine splenic arteries. The solder was activated using a laser diode emitting at 670 nm. The burst pressures of the anastomoses were tested, and the results analysed as a function of MB concentration and absorption. In addition, the relationship between MB concentration and absorption was examined.**

Results: **A dose-response relationship was found between the measured absorption of the solder and the burst pressure of the anastomoses formed. Burst pressures exceeding physiological levels were found. Changes in MB concentration revealed a marked negative deviation from Beer's law at 670 nm, owing to the monomer-dimer-trimer equilibria.**

Conclusion: **PSA with MB solder is able to form high-quality end-to-end anastomoses, with immediate burst pressure profiles similar to those previously described for sutured [Quigley et al., Microsurgery 1985;6: 229], lasered [Quigley et al., Microsurgery 1985;6:229], and soldered anastomoses [Small et al., J Clin Laser Med Surg 1997;15:205]. The relationship between burst pressure strength and chromophore absorption is discussed. Lasers Surg. Med. 26:323–329, 2000.**

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Key words: absorbance; chromophore; laser

INTRODUCTION

Laser-activated tissue solders have been produced for the anastomosis of blood vessels [1,2], with a view to performing sutureless anastomoses. Solders were developed in response to the

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*Correspondence to: Jeremy F. Birch, MD, Department of Surgery, RK CSB, Leicester University, Leicester LE7 5WW, UK. E-mail: jezmd@globalnet.co.uk

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high rate of aneurysm formation caused by laser power alone [3,4]. Jain and Gorisch [5], by using an argon ion laser, showed that laser irradiation of a vein or muscle at the site of the join could reinforce the anastomosis. However, it was not until later that chromophores such as fuschin [6,7], fluorescein isothiocyanate (FITC) [8], or indocyanine green (ICG) [9] were used in an attempt to reduce the required incident laser power density by selectively absorbing the laser wavelength and converting the incident photon energy to heat. Further evolution in this field involved the addition of fibrinogen [1] and later albumin [10] as structural elements to further reduce damage to proteins in the tunica media and adventitia.

Methylene blue (MB) has been suggested as a chromophore in a laser-activated solder [10]. The same report describes no change in the absorbance profile of the methylene blue on addition of up to 50% human albumin. However, results from methylene blue-soldered vascular anastomoses have not been reported.

The chromophore in a protein solder is considered to act primarily as a heat generator, absorbing the incident photon energy, becoming excited and generating thermal energy. This in turn causes protein solidification. The mechanism of this reaction may include cross-linking, disulphide bridge formation, or unraveling and tangling of the protein chains. For those chromophores with appreciable triplet state quantum yields, there may also be a component involving type I or II photosensitised production of singlet oxygen leading to the oxidation of albumin residues [11]. There may also be the possibility of photochemical reaction of the chromophore with solder components However, little is known of the precise nature of the cohesive and adhesive bonds formed with laser-activated solder.

Soldered vascular anastomoses have been reported by using other chromophores but little is known of the optimal photochemical constituents of such a solder. There are some indications of the optimal protein contents of a solder [12,13], but the effects of changing chromophore absorbance are unknown. With this in mind, we set out to investigate the effect of changing variables in the composition and manufacture of a solder based on the chromophore methylene blue with porcine albumin as the structural protein.

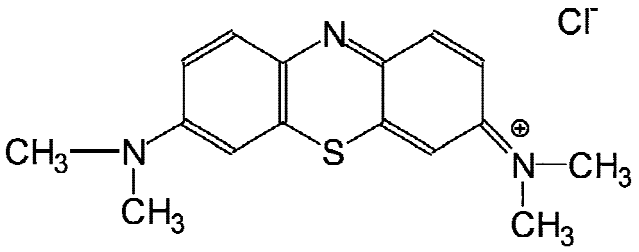

Fig. 1. The structure of methylene blue.

METHYLENE BLUE

Chemistry and Photochemistry

$MB^+ Cl^-$ was first described by Caro [14] in 1877. It is a member of the thiazine group of dyes (Fig. 1) and has a relative molecular mass of 373.9. MB absorbs strongly in the visible region of the spectrum (Fig. 2) with an extinction coefficient of $7 \times 10^5 dm^3 mol^{-1} cm^{-1}$ at 664 nm in plain aqueous solution. At low concentrations ($\approx < 10^{-6}$ mol/dm^3), it exists in the monomeric form with a peak absorption at 664 nm. As the concentration is increased, dimers and higher aggregates are formed, the dimers having an absorption maximum at 615 nm. This finding is seen at modest concentrations as a shoulder in the absorbance profile (Fig. 2).

In 1961, Usui et al. [15] found that methylene blue could be photoreduced to the colourless "leuco" form after irradiation with visible light. This leuco form can be oxidised back to MB by atmospheric oxygen, although this reaction is not quantitative and is accompanied by a slight permanent degradation of the dye. A number of different reaction mechanisms have been proposed for the photoreduction of methylene blue [16–18]. Kato et al. [16] proposed that the semi-reduced species was generated by the reaction with the excited monomer triplet with the ground state monomer. Danziger et al. [17] presented a scheme based on the irradiation of MB monomer or dimer, resulting in the formation of a dimeric charge transfer species, leading to the formation of leuco MB (MBH) or regeneration of the ground state. Kamat and Lichtin have suggested a mechanism whereby an electron is ejected from excited MB. This electron becomes solvated and reacts with the dye and hydrogen, forming semireduced MB ($MBH^{+\cdot}$). Semireduced MB can then revert to ground state MB and leuco MB or combine with semioxidised MB ($MB^{2+\cdot}$), giving 2MB.

It is this photobleaching reaction, which

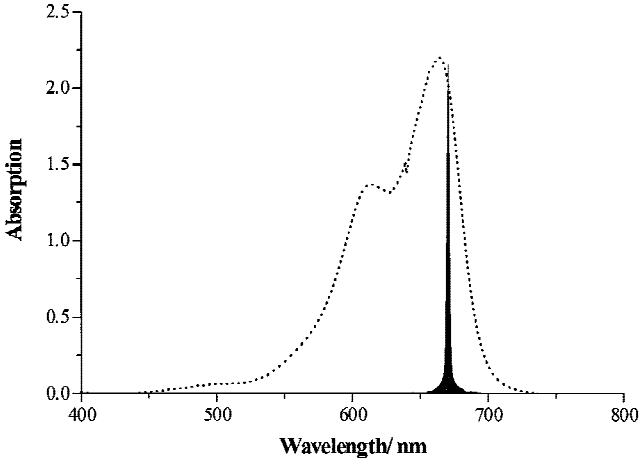

Fig. 2. Absorption spectrum of methylene blue with laser diode emission profile.

makes methylene blue an attractive chromophore for incorporation into a laser-activated solder. By design, we have provided the potential for not only a visual end-point to laser irradiation, but bleaching at the irradiation wavelength results in less incident light being absorbed with consequent protection from over-irradiation and thermal damage to the tissue.

Pharmacology and Clinical Use

Methylene blue was described as the first synthetic antimalarial agent by Ehrlich in 1891 [19]. MB has since been described as a nitric oxide synthetase inhibitor [20], a cGMP inhibitor [21], it also shows anti-muscarinic and anti-cholinergic effects [22]. Nitric oxide synthetase inhibition has been used in the treatment of refractory hypotension in septic shock, and anaphylaxis [23–26]. These latter effects were studied in experimental canine models of endotoxic shock, showing significant changes in both pulmonary and systemic arterial pressure after infusion of MB at levels of 2–10 mg/kg [24,27,28].

MB is also used clinically as a vascular marker. Barber et al. [29] reported that the application of methylene blue to the exterior of saphenous vein, as practiced in coronary artery by-pass graft procedures, may eliminate acetylcholine-induced relaxation, mediated by nitric oxide. Bentz et al. [30] agreed with these findings of reduced vascular relaxation, and although increased platelet deposition was claimed, the results were shown not to be statistically significant.

Other clinical uses of MB include the localisation of parathyroid glands [31], the eradication of renal stones [32,33], the treatment of eczema herpeticum [34,35], the treatment of methaemaglobinaemia (e.g., in dapsone poisoning) [36,37] and in addition MB has been patented as an anti-HIV agent [38].

There is some evidence that the injection of large quantities of MB into the intrathecal space to locate the cause of cerebrospinal fluid rhinorrhoea has caused progressive paraplegias and other neurological deficits [39,40]. Other adverse reactions include the induction of jejunal atresia [27] and haemolysis [41,42] after intra-amniotic injection, as well as haemolysis in conditions of unstable haemoglobin levels [43].

MATERIALS AND METHODS

Solder Preparation

The solder was prepared by using reconstituted dried ingredients. Porcine albumin powder (Sigma Aldrich Chemicals) and methylene blue powder (Sigma Aldrich Chemicals) were mixed together and hydrated with "Water for Injection" BP (Phoenix Pharmaceuticals, UK). The resultant solution was mixed for 30 minutes and left to stand for 2 hours. The final albumin concentration was kept constant at 41% w/w, whereas the absorption of the solder was varied by changing the MB concentration.

Activating System

Activation was performed by using a laser diode system (Laser Module–HPM250/3139, Laser 2000, Ringstead, Northants, UK) coupled to a silica optic fibre (50-μm core diameter) at a wavelength of 670 nm, 180 mW power, and a focused spot diameter of 1 mm at 40 mm. Laser power was measured by using a Coherent power meter (Model 210, Coherent).

Absorption Measurement

Absorption spectra of undiluted solder were recorded in a 100-μm pathlength cell by using a xenon arc lamp as the illumination source and a gated intensified photodiode array detector (EG&G Princeton Applied Research). The solder was irradiated at chromophore concentrations of 0.15–0.41%. The resultant plot of chromophore concentration against absorbance (Fig. 3) was used to determine the absorbance of the solders

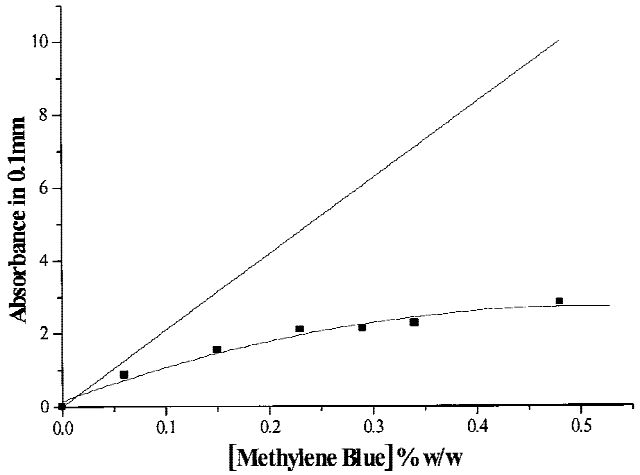

Fig. 3. Solder absorbance as a function of chromophore concentration.

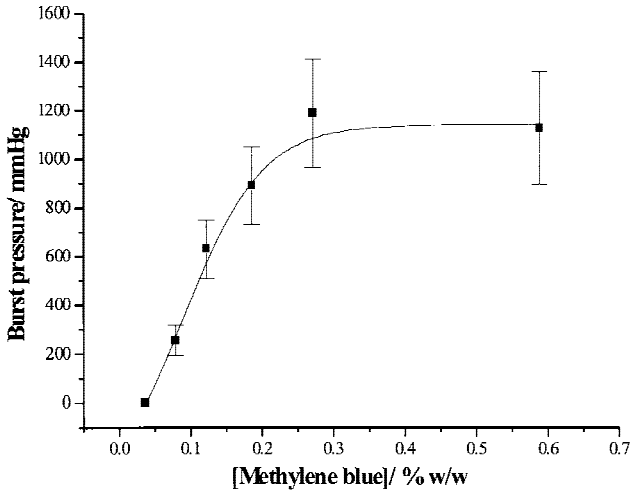

Fig. 4. The burst pressure as a function of solder concentration.

used to anastomose vessels. The spectral profile of the laser diode was measured by using the gated photodiode array system. The use of this apparatus has previously been described [44].

Anastomosis and Burst Pressure Testing (BPT)

Porcine splenic arteries (2–4 mm) were harvested, cleaned, and sectioned; two stay sutures were applied (8/0 ethilon). The vessel was secured on a fenestrated needle, and the stay sutures were used to appose the vessel edges. Volumes of 2–3 μl of solder per side were used and activated with the laser. This procedure was repeated as a second layer was applied. The solder was irradiated for 5 seconds per 1 mm (±100 μm) spot, and chromophore fading was observed as the end point. Typically the number of spots on each side of the vessel was approximately 5, with a small area of overlap between each spot.

The completed anastomosis was pressure tested by using a syringe driver, pressure transducer (0–30 psi) (RS Components, UK), and PC. The needle and vessel were mounted between the transducer, and the syringe pump and the PC were set to acquire data. The vessel was observed for signs of leakage and the maximum pressure was recorded and plotted. Side branches occasionally leaked, and these were occluded by ligation. This process was repeated six times for each concentration of chromophore, and the results were tabulated and plotted (Origin™, Microcal, UK).

RESULTS

The burst pressure obtained as a function of both concentration and absorbance at 670 nm are shown in Figures 4 and 5, respectively. The results of the measurement of absorbance versus chromophore concentration are shown in Figure 3. This figure shows the linear concentration-absorbance curve, calculated by extrapolation to infinite dilution, and the measured values of absorbance at the stated concentrations. Clearly, there is a significant deviation from linearity as a result of aggregate formation, which can clearly be observed in the spectrum.

A sigmoid "dose-response" type curve is seen to changes in concentration and absorption of the solder, with a plateau after a chromophore concentration of 0.0123 mol dm^3 (Fig. 4), corresponding with an absorbance of approximately 2 at 670 nm or approximately 99% of the incident energy absorbed.

The burst pressures achieved in this study show levels not previously recorded in comparable experiments (1,188 ± 222 mmHg), indicating that the potential strength of soldered vascular anastomoses is greater than previously anticipated.

DISCUSSION

Despite the great deal of interest shown in laser vascular anastomoses for over a decade, the data relating to the use of solders is limited to a few publications [45,46]. Vascular anastomosis is one of the commonest and most technically demanding surgical procedures to undertake, and lasers offer the potential for fast, sutureless anastomoses, with reduced vessel trauma and good long-term patency. Initial work, although show-

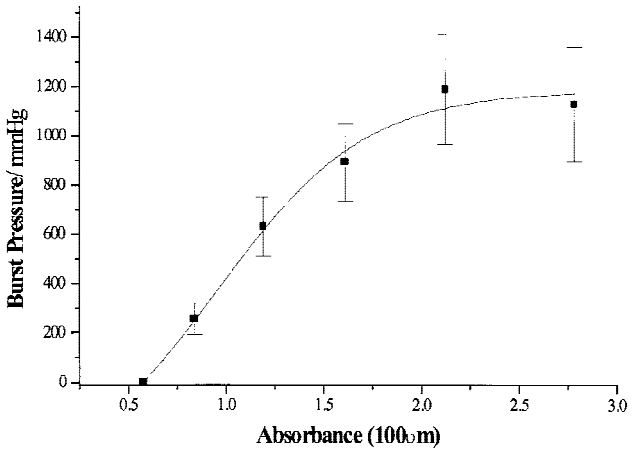

Fig. 5. Burst pressure as a function of solder absorbance.

ing encouraging anastomotic times and patency, revealed a high rate of aneurysm formation and thermal damage [3]. Since that time, efforts have concentrated on controlling laser power, to stay within the "therapeutic window" that both allows an adequate anastomosis to be formed while preventing thermal damage. Strategies have included the use of protein solders [47], chromophores [8], and temperature-controlled feedback systems [48].

Chromophore contained in solders include fuschin, eosin [7], ICG [9], and FITC [49] for applications such as urologic reconstruction, choledochotomy repair, and skin closure. These chromophores, although showing a good absorption match with laser emission, show no photobleaching, continuing to absorb long after solder polymerisation. ICG does show a change in colour [50], but this provides little in the way of visual indications of complete solder activation. FITC has also been investigated because it undergoes a reduction in fluorescence with laser exposure. However, MB displays photobleaching, giving the surgeon visual feedback, as well as an absorption "switch," preventing overexposure of tissue.

Soldered vascular anastomoses in a canine model have been described recently [46] where a 50% albumin solder without chromophore was used. The time of anastomosis was significantly reduced relative to sutured controls, but thermal damage was reported through 30% of the arterial wall. In this study, we describe the use of a methylene blue-based solder activated by a laser diode system at 670 nm. This combination of chromophore and laser provides a good correlation between the absorption and emission (Fig. 2) to produce anastomoses capable of withstanding supraphysiological pressures.

A sigmoid "dose-response" type curve is seen for concentration against burst pressure (Fig. 4). However, the relationship with absorption shows a flatter curve and the increase in anastomotic strength rises more evenly (Fig. 5). The explanation for this finding lies in an understanding of the behaviour of MB in a solder. Calculations suggest that solder applied to the anastomosis has a thickness of 0.1 mm. Measurement of absorbance (at 670 nm; 0.1 mm pathlength) against MB concentration (Fig. 3) shows a significant deviation from the Beer-Lambert law at high concentrations. This deviation is thought to be due to the well-known monomer-dimer-trimer equilibria observed for MB [51], and indeed at higher concentrations, dimeric and trimeric forms dominate the equilibrium mixture. Because only the monomeric form absorbs significantly at 670 nm, the change in the relative concentrations between these molecules affects the absorption of the solder used in this study [51].

Figure 5 shows that the rate of increase in burst pressure with increasing absorbance slows as absorbance becomes greater. This finding is because a finite temperature must be reached before solder curing is achieved. Once the absorbance achieves a level where essentially all of the incident photons are absorbed, a maximum initial heating rate will be achieved. However, even beyond the level where 99% of photons are absorbed (an absorbance of 2), there is still some increase in burst pressure with increasing concentration. This can be explained by the photochemical reaction of MB and the formation of the "leuco" form, with loss of absorption at 670 nm. Hence, at lower concentrations, this reaction results in a rapid decrease in the proportion of photons absorbed. At higher concentrations, although the same rate of concentration loss is expected, because there is a greater initial absorbance, there is a significant irradiation time before there is a significant decrease in the proportion of incident photons absorbed. Consequently, the time for which heating is sustained is greater.

This finding indicates that in order for optimal burst pressures to be achieved, sufficient thermal energy must be generated. The period required will be a function of the absorption of the solder and the state of hydration of the vessel, which will alter heat capacity and heat conduction at the anastomosis. Dehydration of the solder is also a significant factor in the curing reaction.

Therefore, the degree of tissue hydration is critical but difficult to control, affecting both the formation of cohesive bonds (solder to solder) and adhesive bonds (solder to adventitia). The optimal conditions for these interactions are poorly understood at present, and further investigation is required to refine solder vascular anastomosis [52].

Attempts have been made to avoid thermal damage by using a temperature controlled feedback system incorporating infrared detectors [48] or thermocouples [53], but these devices can only give an estimate of the surface temperature reached. They are also difficult to align because of the small size of the anastomotic area. By using a system based on a photochemically bleaching chromophore, such as that described here, temperature rise is potentially limited as photobleaching will reduce absorption and limit the heat build-up, preventing overexposure. Careful control of the chromophore concentration will allow control over the peak temperature reached, and over the length of time for which this is sustained, although no attempt has been made to quantify this effect in this study.

MB has been used in this study to produce a novel tissue adhesive that provides a visible end point and potentially a self-regulating absorbance switch. This solder is able to produce anastomoses capable of withstanding high pressures, and is produced with the intention of being both surgeon friendly and having the potential to eliminate thermal damage by making use of chromophore fading.

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