Photodynamic Tissue Adhesion with Chlorin<sub>e6</sub> Protein Conjugates

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PURPOSE. To test the hypothesis that a photodynamic laser-activated tissue solder would perform better in sealing scleral incisions when the photosensitizer was covalently linked to the protein than when it was noncovalently mixed.

METHODS. Conjugates and mixtures were prepared between the photosensitizer chlorin<sub>e6</sub> and various proteins (albumin, fibrinogen, and gelatin) in different ratios and used to weld penetrating scleral incisions made in human cadaveric eyes. A blue-green (488–514 nm) argon laser activated the adhesive, and the strength of the closure was measured by increasing the intraocular pressure until the wound showed leakage.

RESULTS. Both covalent conjugates and noncovalent mixtures showed a light dose-dependent increase in leaking pressure. A preparation of albumin chlorin<sub>e6</sub> conjugate with additional albumin added (2.5 protein to chlorin<sub>e6</sub> molar ratio) showed significantly higher weld strength than other protein conjugates and mixtures.

CONCLUSIONS. This is the first report of dye–protein conjugates as tissue solders. These conjugates may have applications in ophthalmology. (Invest Ophthalmol Vis Sci. 1999;40:3132–3137)

Alternative methods to the traditional mechanical means of closing incisions, wounds, and anastomoses have recently received attention. These may be divided into three groups: first, biological glues such as fibrin sealant and gelatin-resorcinol glue; second, a technique known as laser tissue welding, which relies on carbon dioxide or Nd:YAG lasers to produce thermal effects to attach tissue surfaces; and third, chromophore-assisted laser welding using protein solders that contain a light-absorbing dye together with a laser that emits the appropriate wavelength light. This pairing is most commonly that of fluorescein and a 532-nm frequency-doubled Nd:YAG laser, or indocyanine green and an 805-nm diode laser. It is generally thought that the energy absorbed by the dye is released into the tissue as heat that then denatures proteins and produces noncovalent bonds between the added protein solder and the tissue collagen. However, depending on the dye used, there may be contributions from photochemical reactions that produce covalent cross-links between protein molecules. These techniques have been used in urology, vascular surgery, neurosurgery, and orthopedics.

Ophthalmologic applications of laser welding with chromophore-assisted protein solder have included sealing cataract incisions and scleral tunnel incisions and bonding synthetic epikeratoplasty lenticules to the cornea. We have previously described preliminary work on a photodynamic tissue adhesive: specifically, the use of a preparation consisting of 18% fibrinogen and riboflavin-5-phosphate (r-5-P), together with argon laser activation to close corneal incisions in cadaveric eyes. The finding that addition of sodium azide to the glue preparation reduces the leaking strength by more than 50% is attributed to quenching of singlet oxygen. This indicates that photodynamic mechanisms may be operating in the formation of covalent protein cross-links. We now report on the use of a chromophore that may prove even more effective in cross-linking proteins by a photodynamic mechanism, namely chlorin<sub>e6</sub> (C<sub>e6</sub>). We have previously reported on the use of covalent conjugates between C<sub>e6</sub> and monoclonal antibodies and poly-L-amino acids for the photodynamic therapy of cancer. Our hypothesis was that covalent conjugates between C<sub>e6</sub> and proteins used as laser-activated solders may form stronger tissue bonds than noncovalent mixtures.

MATERIALS AND METHODS

C<sub>e6</sub> was obtained from Porphyrin Products (Logan, UT), N-hydroxy succinimide (NHS), dicyclohexylcarbodiimide, bovine fibrinogen, bovine serum albumin (BSA), and gelatin were from Sigma (St. Louis, MO). Frozen, nonpreserved, human cadaveric eyes were obtained from the Illinois Eye Bank (Chicago).

Preparation of Conjugates

All reactions were performed in the dark at room temperature. The NHS ester of C<sub>e6</sub> was prepared by reacting 1.5 equivalents of dicyclohexylcarbodiimide and 1.5 equivalents of NHS with 1 equivalent of C<sub>e6</sub> in dry dimethyl sulfoxide (DMSO) for 24 hours and was frozen in aliquots for further use. The concentration of the C<sub>e6</sub>-NHS in DMSO was 100 mM. Proteins were...
dissolved in 0.1 M NaHCO₃ buffer (pH 9.3). For BSA the concentration was 500 mg/ml, for fibrinogen 100 mg/ml, and for gelatin 200 mg/ml. A fivefold molar excess of **Ce₆-NHS** ester in DMSO was added to the protein solution, which was allowed to stand overnight. The crude conjugate solution was then dialyzed twice against 5 l phosphate-buffered saline (PBS) to remove unconjugated **Ce₆** and DMSO. Mixtures of **Ce₆** and proteins were prepared by dissolving **Ce₆** in 0.1 M NaOH to form a 100-mM solution, adding the requisite amount to the protein solution in PBS and neutralizing with 0.1 M HCl. Conjugates were characterized by absorption spectroscopy after suitable dilution in PBS.

**Welding Procedure**

To measure leaking pressures, an 18-gauge butterfly needle was connected by plastic tubing to a water bottle. The inner pressure in the bottle was controlled by a hand-pumped sphygmomanometer. Nonpreserved cadaveric eyes were defrosted in room-temperature water. The butterfly needle was inserted in the vitreous cavity through equatorial sclera, and the eye was pressurized to 25 to 30 mm Hg. The corneal epithelium was then removed at the wound site, and a caliper set on 5 mm used to mark the extent of the incision on equatorial sclera, perpendicular to the limbus. All incisions were placed equidistant from the limbus, and areas of blue or thin sclera were avoided. A perpendicular perforating incision was made with a 15° blade. Incisions were made into the vitreous cavity and extended to the full length using Vannas scissors. The glue was applied in a thin layer to the surface of the wound with a tuberculin syringe and a 30-gauge needle. A small amount was injected within the wound. Argon blue-green laser (488–514 nm; Spectrum K3, HGM Medical Lasers, South Salt Lake City, UT), at a setting of 0.6 W, 2-mm-diameter spot size, was applied to the wound for 60 to 120 seconds in a continuous back and forth manner using a handheld fiberoptic probe. The exact power output of the fiber was measured using a power meter (model 210; Coherent, Palo Alto, CA). Argon laser goggles (Glendale Protective Technologies, Lakeland, FL) were worn by the operator, which allowed viewing of the fluorescence from the **Ce₆** (emission 670 nm). To set the remaining adhesive on the scleral surface, which surrounded the wound, additional laser was applied until loss of fluorescence of the dye, which took another 30 to 45 seconds.

**Leaking Pressure**

Leaking pressures were then measured using the sphygmomanometer, which was increasingly pressurized in approximately 10-mm Hg increments. Leaking pressure was recorded the moment the wound leaked air or fluid. This procedure was used in all eyes.

**RESULTS**

The reaction sequence used to attach the **Ce₆** molecules to the proteins covalently is shown in Figure 1. The conjugate between BSA and **Ce₆** could be prepared easily and dialyzed to give a viscous dark green solution. Conjugates, which contained approximately 6 mM BSA (400 mg/ml), had the appropriate consistency and viscosity for using as solders in incisions. The conjugate between gelatin and **Ce₆** could not be prepared in a high enough concentration to yield a sufficiently viscous solution. The conjugate between fibrinogen and **Ce₆** was substantially aggregated and unsuitable for use as solder. To explore the effect of these proteins on the weld strength, gelatin was added to BSA-**Ce₆**, and fibrinogen was mixed with BSA-**Ce₆** and with free **Ce₆**. The compositions of the conjugates and mixtures that were used as solders are shown in Table 1. The absorption spectra of three of the mixtures and conjugates between BSA and **Ce₆** are shown in Figure 2.

The results from the welding experiments and determinations of the leaking pressures are shown in Table 2. The total energy delivered varied from 24 to 57 J (60–120 seconds' exposure at powers ranging from 0.4 to 0.52 W). At first, we

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*FIGURE 1.* Reaction scheme used to prepare covalent protein **Ce₆** conjugates. DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DMSO, dimethyl sulfoxide.
tried to compare the adhesive strength of the welds produced by BSA-c
t conjugate and BSA + c
t mixture in which the molar ratios of protein to c
t were roughly one to one. However the leaking pressures obtained were low, and when we added more BSA to the mixture of BSA + c
t to attain a molar ratio of 2.5:1 protein to c
t, the leaking pressure showed a marked increase (Table 2). Considering this, we then modified the protein-to-c
t ratio to at least 2.1 protein to c
t in the remaining preparations. We compared the results with the preparation we had previously used when (r-5-P) and fibrinogen were used at a protein-to-r-5-P ratio of 0.16.

The BSA + c
t (2.5:1) was compared with the BSA-c
t with added BSA, which raised the protein ratio to 4:1. Also investigated was a mixture of BSA-c
t and gelatin that had an overall protein-to-c
t ratio of 4:1. The leaking pressure was measured as a function of applied fluence for these three solder preparations, and the results are shown in Figure 3. A total energy-dependent increase in leaking strength was seen for all preparations, and the results are shown in Figure 3. A total energy-dependent increase in leaking strength was seen for all three solders,

The leaking pressure was measured as a function of applied fluence for these three solder preparations, and the results are shown in Figure 3. A total energy-dependent increase in leaking strength was seen for all three solders, and the order of weld strength was found to be BSA-c
t + BSA > BSA + c
t > BSA-c
t + gelatin. Also investigated as solders were mixtures of fibrinogen with c
P (2.1) and with BSA-c
t (2.5:1). These solders performed particularly poorly, with a leaking pressure of only 30 mm Hg. We compared these results with the preparation we previously described consisting of fibrinogen and r-5-P at a ratio of 0.16 protein to r-5-P. The leaking pressure we obtained was similar to that we had previously reported (Fig. 3). These various mean leaking pressures were tested for significance with a two-tailed unpaired Student’s t-test. The strength of the weld from BSA-

c
t + BSA was significantly greater than that from fibrinogen + r-5-P (P < 0.05), BSA + c
t (2.5:1; P < 0.05), BSA-c
t and gelatin (P < 0.0005), whereas there was no significant difference between the strengths from fibrinogen + r-5-P and from BSA + c
t (2.5:1), whereas both were significantly stronger than BSA-c
t + gelatin (P < 0.05).

During the welding procedure there was a visually detectable change in the appearance of the solder, which at first was dark green and became brownish green. Care had to be taken at the ends of the weld where the movement of the handheld fiberoptic probe naturally slowed down in preparation for changing direction. This meant that the center of the incision received less light than the ends and that leakage of the wound started at this point. The consistency of the solder after welding was firm and smooth, and the tissue showed no thermal damage. This could easily be seen if the total energy or the power at which it was delivered was too great. The edges of the wound retracted and started to wrinkle, and the weld failed, because the wound edges were no longer in apposition. Any solder resting on the cornea that did not receive laser exposure continued to have a liquid, nonadhesive consistency.

**DISCUSSION**

Tissue welding with the aid of laser-activated solders is a very attractive concept, because it would allow sutureless surgery, as well as repair of certain wounds that are difficult or impossible to close by standard suture techniques. This is of partic-

<table>
<thead>
<tr>
<th>Composition</th>
<th>Eves</th>
<th>Total Energy Range</th>
<th>Mean Leaking Pressure</th>
<th>Mean Leaking Pressure/Total Energy Ratio</th>
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</table>
| BSA-c
t conjugate           | 7    | 39–46              | 78.0 ± 11.9           | 1.75 ± 0.26                            |
| BSA + c
t mixture (1:1)     | 3    | 24–46              | 63.0 ± 24.3           | 1.77 ± 0.38                            |
| BSA + c
t mixture (2.5:1)   | 7    | 24–46              | 127.1 ± 13.4          | 4.04 ± 0.52                            |
| BSA-c
t conjugate + BSA     | 8    | 24–57              | 207.1 ± 11.1          | 5.59 ± 0.24                            |
| BSA-c
t conjugate + gelatin| 11   | 26–53              | 101.8 ± 14.2          | 2.71 ± 0.47                            |
| Fibrinogen + c
t mixture   | 3    | 24–46              | 39.6 ± 12.5           | 1.1 ± 0.34                             |
| BSA-c
t conjugate + fibrinogen| 3  | 24–53              | 35.8 ± 11.8           | 0.93 ± 0.28                            |
| Fibrinogen + r-5-P          | 6    | 24–46              | 139.5 ± 12.3          | 4.44 ± 0.53                            |

Incisions were closed by welding using the solder composition and total energies specified and leaking pressure measured with a sphygmomanometer as described. Total energy is in joules, and leaking pressure is in millimeters of mercury.
ular importance in the field of ophthalmology, because sutures, staples, and clips all involve additional tissue injury, and a foreign body response that can lead to increased inflammation, scarring, and stenosis. In addition, gluing or welding tissue may reduce wound slippage and render the wound impermeable to microorganisms. The ideal material to use as a glue or solder should be strong, effective, nontoxic, biodegradable and available in a sterile preparation. For the solder preparation to be effective, it should also have a sufficiently high viscosity to enable it to stay in contact with the wound during welding, while allowing it to be delivered through a narrow-gauge needle. The ideal consistency would therefore be thixotropic (i.e., a material that has lower viscosity at higher shear stresses).

There are many potential applications of this technology in ophthalmology including the repair of leaking filtering blebs, corneal ulcers, and scleromalacia perforans. It may be used in construction of a temporary tarsorrhaphy, and the reinforcement of sclera in patients with thin sclera or staphyloma.

Alternative tissue adhesives have drawbacks. Cyanoacrylate glues, which have been most frequently used in ophthalmology, can be toxic, causing inflammatory reactions and are nonbiodegradable. Fibrin sealants are not particularly effective, form bonds of insufficient strength, present the possibility of viral infection if prepared from pooled human plasma, and may inhibit wound healing. Resorcinol gelatin sealants can damage tissue, because they contain formaldehyde. Laser-activated tissue solders are more promising, allowing safe preparation and sterilization of the material, because it is activated only under laser illumination and is thought unlikely to lead to tissue toxicity. It is therefore necessary to investigate the structure and composition of both the solder and dye and to attempt to optimize performance in different tissue welding applications.

It is quite clear that laser tissue welding without added dye must proceed through a purely thermal mechanism, whereby the edges of the collagen are partially "unraveled" and can then recombine to form noncovalent bonds. It was thought that dye-assisted welding with protein solders also proceeded through a thermal mechanism, with the chromophore-absorbing energy, releasing it as heat, denaturing the protein in the solder and forming noncovalent bonds to the tissue. However, recent results with the two dyes most commonly used for tissue welding, fluorescein and indocyanine green, have produced evidence that photochemical processes occur as well. It has been reported that fluorescein-dextran in the rat mesentery lymphatics when illuminated produce changes that could be attributed to singlet oxygen. Experiments with indocyanine green in vitro have shown that it has a triplet yield of 0.11, and singlet oxygen can be detected.
by time-resolved luminescence techniques.\textsuperscript{35,36} Our previous report\textsuperscript{16} on laser welding with a biologic tissue glue consisting of 18% fibrinogen with 2.6 mg/ml r-5-P showed reduction of the weld strength in the presence of azide which is evidence of singlet oxygen involvement in the weld formation. We therefore decided to test a photosensitizer which has known triplet and singlet oxygen quantum yields, namely $c_{\text{es}}$. $C_{\text{es}}$ has been extensively investigated as a photosensitizer for photodynamic therapy both as the free dye\textsuperscript{37} and conjugated to proteins,\textsuperscript{38} macromolecules,\textsuperscript{18} and particles.\textsuperscript{39}

Because there is some likelihood that covalent bond formation mediated by a photodynamic process in a protein solder improves weld strength, we decided to test this hypothesis by comparing covalent conjugates between the protein and $C_{\text{es}}$ with noncovalent mixtures. We reasoned that a photosensitizer molecule already joined to the protein would be more likely to form a bond between that protein and a neighboring protein molecule than a photosensitizer that had to be close to two protein molecules at the same time. Our finding that the strength of the weld formed by the BSA-$C_{\text{es}}$ conjugate was significantly stronger than that formed by the noncovalent mixture confirmed our hypothesis and additionally showed that a photosensitizer widely thought to proceed through a type II mechanism could form satisfactory tissue welds when applied in protein solder.

How is the dramatic improvement in weld strength when the protein-to-$C_{\text{es}}$ ratio is increased to be explained? Presumably, intermolecular cross-links between albumin molecules are more likely to form between one conjugate molecule and one unconjugated albumin molecule than between two conjugate molecules. $C_{\text{es}}$ is usually thought to act as a photosensitizer by transferring energy from the triplet state to the ground state of molecular oxygen, producing the excited singlet oxygen molecule, a process known as type II photosensitization.\textsuperscript{40} Singlet oxygen can then react with certain amino acids in proteins, particularly histidine, tryptophan, tyrosine, cysteine, and methionine.\textsuperscript{41} One mechanism that has been elucidated for the formation of intermolecular protein cross-links is the reaction of oxidized histidine with free $\epsilon$-amino groups of lysines on neighboring proteins,\textsuperscript{52} but it is recognized that other mechanisms must operate as well. There is another possible photo-oxidation pathway involving electron transfer from the photosensitizer triplet state producing either a radical cation or a radical anion, which is known as type I photosensitization.\textsuperscript{43} These radical ions can then react further with oxygen producing carbon- and oxygen-centered radicals and superoxide anions.\textsuperscript{44} A mechanism for the radical mediated cross-linking of proteins involves the formation of dityrosine\textsuperscript{45} by phenolic coupling of tyrosine residues on neighboring chains. However, it must be emphasized that further experiments are necessary to confirm the formation of covalent protein cross-links.

Many questions remain to be answered in this photodynamic tissue solder procedure. Can covalent protein cross-linking be demonstrated by polyacrylamide gel electrophoresis? Are there albumin dimers? Is there evidence of albumin-collagen cross-linking? Can the relative contributions to the mechanism from type I and type II photoprocesses be determined? Although the green-blue argon laser is frequently used in ophthalmology, it can be readily seen from Figure 2 that the wavelengths are suboptimal for excitation of $c_{\text{es}}$. Will the use of a red (665-nm) diode laser lead to improved weld strength, possibly by allowing higher powers to be used without leading to thermal damage? The procedure should be repeated with fresh eyes to control for any effects of freezing on the structural integrity of the sclera. For this procedure to be applied in vivo, it is necessary to establish the integrity of the weld in eyes subject to natural secretions and to show it can actually induce a long-lasting wound closure with resistance to the enzymatic degradation associated with scleral wound healing. It is also necessary to investigate to what extent this laser-activated adhesive induces an inflammatory response, and immunologic issues may require investigation.

\textbf{References}