Video Article

Fabrication and Application of Rose Bengal-chitosan Films in Laser Tissue Repair

Antonio Lauto[1], Marcus Stoodley[2], Matthew Barton[1], John W. Morley[1], David A. Mahns[1], Leonardo Longo[3], Damia Mawad[1]

[1]Bioelectronics and Neuroscience (BENS) research group, University of Western Sydney, NSW Australia

[2]Australian School of Advanced Medicine, Macquarie University, NSW Australia

[3]School of Medicine, University of Siena, Italy

Correspondence to: Antonio Lauto at a.lauto@uws.edu.au

URL: https://www.jove.com/video/4158
DOI: doi:10.3791/4158

Keywords: Bioengineering, Issue 68, Photochemical tissue bonding, tissue repair, nerve anastomosis, sutureless technique, chitosan, surgical adhesive

Date Published: 10/23/2012

Citation: Lauto, A., Stoodley, M., Barton, M., Morley, J.W., Mahns, D.A., Longo, L., Mawad, D. Fabrication and Application of Rose Bengal-chitosan Films in Laser Tissue Repair. *J. Vis. Exp.* (68), e4158, doi:10.3791/4158 (2012).

Abstract

Photochemical tissue bonding (PTB) is a sutureless technique for tissue repair, which is achieved by applying a solution of rose bengal (RB) between two tissue edges[1,2]. These are then irradiated by a laser that is selectively absorbed by the RB. The resulting photochemical reactions supposedly crosslink the collagen fibers in the tissue with minimal heat production[3]. In this report, RB has been incorporated in thin chitosan films to fabricate a novel tissue adhesive that is laser-activated. Adhesive films, based on chitosan and containing ~0.1 wt% RB, are fabricated and bonded to calf intestine and rat tibial nerves by a solid state laser (λ=532 nm, Fluence~110 J/cm^2, spot size~0.5 cm). A single-column tensiometer, interfaced with a personal computer, is used to test the bonding strength. The RB-chitosan adhesive bonds firmly to the intestine with a strength of 15 ± 6 kPa, (n=30). The adhesion strength drops to 2 ± 2 kPa (n=30) when the laser is not applied to the adhesive. The anastomosis of tibial nerves can be also completed without the use of sutures. A novel chitosan adhesive has been fabricated that bonds photochemically to tissue and does not require sutures.

Video Link

The video component of this article can be found at https://www.jove.com/video/4158/

Protocol

1. Chitosan Adhesive Preparation

1. The chitosan powder is soluble in acetic acid solution; for the preparation of a stock solution of acetic acid (2% v/v), add 10 ml glacial acetic acid to 490 ml deionized water (DI-H_2O).
2. For the preparation of a stock solution of Rose Bengal (RB) (0.01% w/v) in acetic acid, weigh 5 mg of RB in a vial wrapped with aluminum foil to avoid photobleaching. Add 0.5 ml of DI-H_2O to dissolve the powder then add 49.5 ml of the acetic acid stock solution (described in section 1.1).
3. For the preparation of chitosan solution (1.7% w/v), weigh 0.85 g of chitosan powder (medium MW, 85% degree of acetylation) and add to 50 ml of RB acetic acid solution. The control was prepared by dissolving the chitosan in acetic acid solution without RB.
4. Add a magnetic stirrer to the chitosan mixture and stir at room temperature. To ensure complete dissolution of the components, stir the chitosan in acetic acid + RB for two weeks and the chitosan in acetic acid for 5 days (control). When chitosan is dissolved, the solution pH increases from ~2.6 to ~3.9. Note that RB is poorly soluble in acidic pH and it requires an increase in the pH of the solution and an extended stirring time to dissolve.
5. Centrifuge the solutions at 3270 x g for 1 hr and collect the supernatant using, for example, a sterile 10 ml syringe. Make sure not to disturb the impurity pellet. During this step, the solution is cleaned from macroscopic impurities and insoluble matter.
6. Inject the (centrifuged) chitosan solution on a flat perpex slab using a sterile syringe. The thickness of the dried film depends on the ratio between the dispensed amount of solution and the surface area over which the solution is spread. Ensure that the solution is free from air bubbles and evenly spread on the slab. To obtain thin films with ~13 μm thickness, inject ~1 ml solution over 12 cm^2 surface area (dimensions~3x4 cm, **Figure 1A**).
7. Cover the Perspex slab with a screen, made of aluminum foil for example, to avoid photobleaching of the solution. Ensure that enough air ventilation is guaranteed to dry the solution under the screen.
8. Leave the solution to dry at room temperature (25 °C) and pressure (1 atm) till the resulting film is insoluble in water and does not swell macroscopically. This is typically achieved in 2 weeks.
9. Detach the films carefully using a thin and flat spatula: these films can be easily peeled off the Perspex slab without damaging them.
10. Measure the thickness of the films using a micrometer.

11. Cut the adhesive in rectangular strips with a clean sharp scissor and place them between sterile glass slides to preserve their flat shape. Wrap the slides in aluminum foil for light shielding and store them at room temperature. Label the adhesives with or without RB as "rose adhesive" or "chitosan adhesive" respectively.
12. The rose adhesive films can be sterilized using ethanol (90%) or with gamma radiation (0.1 KGy/h for 24 hr)[4,5].

2. Adhesive Optical Attenuation

1. Fix the rose adhesive inside a quartz cuvette.
2. Record its attenuated spectrum in the range of 400 - 800 nm using a dual-beam spectrophotometer[4].
3. Assuming the validity of Beer's law, calculate the attenuation length of the adhesive as follows: $I = I_0 e^{-Ax}$ where I_0 is the incident beam intensity, 1/A is the attenuation length, and x is the film thickness.
4. Repeat steps 2.1 to 2.3 for the chitosan adhesive to serve as a control.

3. *In Vitro* Application: Laser Tissue Bonding on Intestine

1. Harvest calf intestine immediately after animal euthanasia and store at -80 °C.
2. Prior to use, defrost and hydrate the tissue by immersing it in DI-H_2O for 10 min.
3. Bisect the tissue into sections (2 x 1 cm) using a #10 blade under an operating microscope (x10). Keep the sections moist using DI-H_2O.
4. Bring the incision stumps close together (end to end). Wipe excess water off the surface using cotton tips.
5. Place a rose adhesive strip (10 x 6 mm) across the incision on the serosa layer with microforceps ensuring full contact with the intestine (**Figure 2**).
6. The tissue adhesion of the rose adhesive is activated by a diode-pumped solid state laser, which is coupled to a multimode optical fiber (core diameter 200 µm)[4].
7. Set the power level of the laser to 180 mW and irradiate the adhesive for ~6 min with a beam spot size of ~5 mm, using the parameters listed in **Table 1**. Spot-irradiate the adhesive ensuring that each spot is irradiated for ~5 seconds before moving the beam to the adjacent spot. This procedure guarantees that the laser beam scans the whole surface area of the adhesive several times.
8. To assess the tissue bonding strength, clamp a sample to a calibrated single column tensiometer (Instron, MA, USA) using mechanical grips. Move the grips at 22 mm/min until the two tissue stumps separate[4].
9. Place a rose adhesive on the tissue as described in step 3.5. Do not irradiate the sample and measure the tissue bonding strength as described in step 3.8. This sample serves as a control.

4. *In Vivo* Application: Tibial Nerve Anastomosis

1. Sedate the Wistar rat with isoflurane/O_2 mixture (4% during induction, 2% thereafter)[5]. Subcutaneously inject Buprenex (0.03 mg/kg) to provide intra-operative analgesic activity and alleviate possible pain or discomfort.
2. The following surgical procedure should be performed under an operating microscope (x10). Make an oblique skin incision about 3 cm long in the dorso-lateral part of the right thigh and expose the tibial nerve with a muscle-splitting approach through the gluteal muscles[2].
3. Partially dissect and trim the adventitia of the tibial nerve using straight micro-scissors and absorb excess water with sterile gauze or cotton tips.
4. Cut the tibial nerve with micro-scissors.
5. Position a sterile chitosan strip (dimensions 5 x 4 mm) underneath the tibial nerve using micro-forceps.
6. Approximate the nerve stumps end-to-end with micro-forceps over the rose adhesive strip.
7. The strip fully adheres to the nerve forming a collar, which can assist with the rotation of the nerve during the laser irradiation of the strip.
8. Activate the rose adhesive with the laser as described in steps 3.6) and 3.7).
9. Trim the redundant rose adhesive from the collar of the operated nerve.
10. Close the muscles and skin with five 3-0 sutures[5].
11. Standard post-operative recovery procedures should be followed including daily inspection for dehiscence or pus formation and analgesic administration, if required.

5. Representative Results

The films that are obtained are bright rose in color, thin and have a smooth surface (**Figure 1**). They are also flexible and can be rolled into tubes of small diameter without causing tears or any other apparent damage (**Figure 1B**). The rose adhesive has two absorption peaks at 530 and 562 nm; the green laser is thus strongly absorbed by the adhesive and the corresponding attenuation length at 532 nm is ~12 µm (**Figure 3**). In contrast, chitosan films without RB attenuates weakly the laser (1/A ~162 µm), likely due to scattering. It appears from the spectra plot that no significant aggregation of RB occurs in the films.

The rose adhesive bonds firmly to the intestine upon laser irradiation (**Figure 2**) achieving a typical maximum load at failure of 0.9 ± 0.4 N (n=30). The adhesive strength was estimated as the maximum load divided by the adhesive surface area, namely, 15 ± 6 kPa (n=30). The non-irradiated rose adhesive bonded significantly less to tissue (2 ± 2 kPa, n=30, unpaired t-test $p<10^6$). The *in-vivo* anastomosis of the tibial nerve was successfully achieved following the application and bonding of the rose adhesive in conjunction with the green laser. One week postoperatively, the operated nerve was in continuity and the repair strength was 17 ± 9 kPa (n=10).

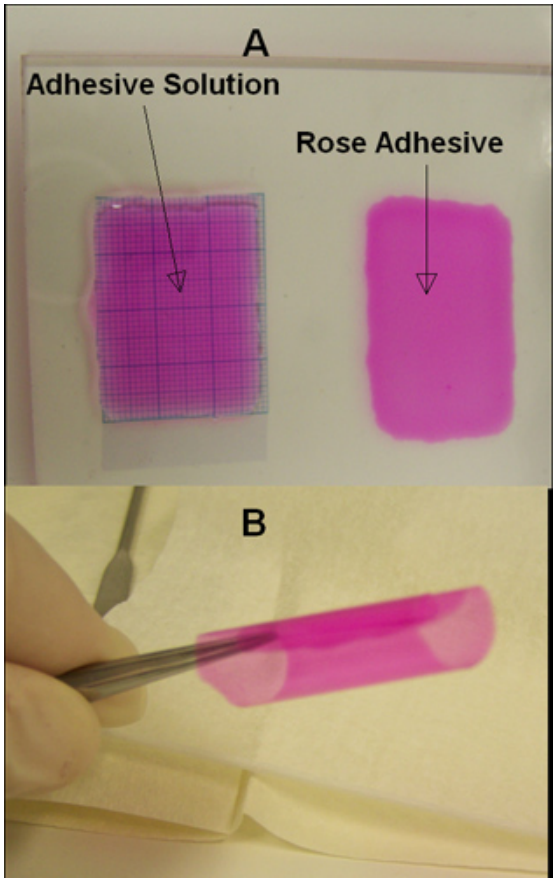

Figure 1. A) The RB-chitosan solution is casted on a flat Perspex slab (slab thickness~5 mm). Graph paper is placed underneath the slab to facilitate the dry-cast step. The water-insoluble film is obtained two weeks after casting. B) The rose adhesive is flexible and can be rolled into a small cylinder.

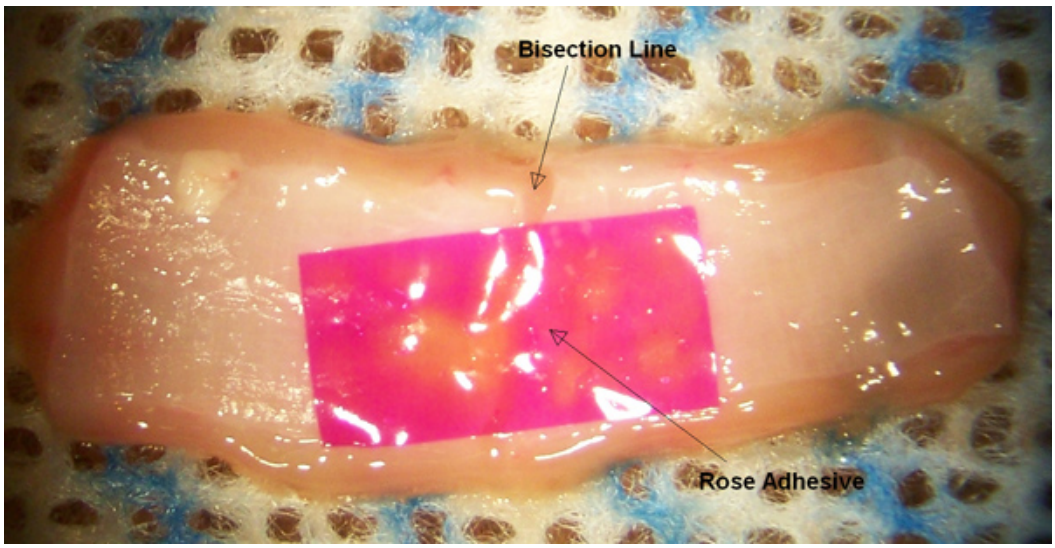

Figure 2. The rose adhesive is bonded to calf intestine after laser irradiation. Uniform irradiation is applied on the RB adhesive during tissue-bonding; however selected spots are shown in this picture to illustrate the photo-bleaching effect of the laser on the adhesive.

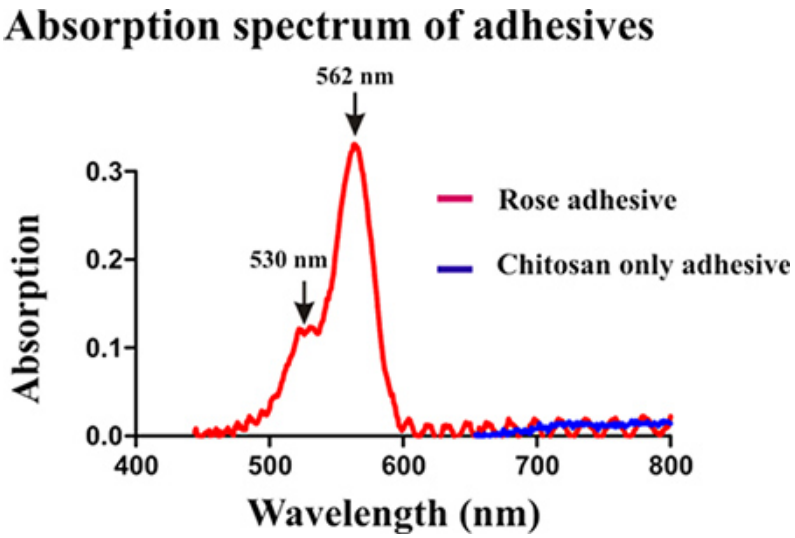

Figure 3. The absorption spectrum (in arbitrary units) of the rose adhesive shows two peaks at 530 and 562 nm. The green laser (λ= 532 nm) is thus strongly absorbed by the adhesive during the laser exposure. The chitosan adhesive without RB absorbs poorly the laser beam.

	n	Area (mm^2)	Power (W)	Time (s)	Fluence (J/cm^2)	I (W/cm^2)	Max Load/Area (kPa)
Adhesive +Laser	30	60 ± 10	0.18 ± 0.03	365 ± 5	~110	~0.9	15 ± 1
Adhesive	30	60 ± 10	NA	NA	NA	NA	2 ± 2

Table 1. Legend. n, sample number; Area, surface area of the rose adhesive (mean ± maximum error); Power, laser power (mean ± maximum error); Time, irradiation time (mean ± maximum error); Fluence, average laser fluence; I, estimated irradiance; Max Load/Area, maximum load at failure of the repaired tissue divided by the adhesive surface area, (mean ± SE).

Discussion

The rose adhesive fabrication is based on a simple dry-cast process, though the dissolution of RB in acidic pH requires prolonged stirring of the chitosan solution. It is important to let the solution dry up until it becomes a water-insoluble film. This happens when the weight water content is ~10% in the dried film[6]. Insoluble films are usually obtained 2 weeks after dry-casting at standard conditions of temperature and pressure (~25 °C and ~1 atm). The mechanism of tissue bonding is not clear yet, although it was observed that RB diffuses from the adhesive into the adjacent tissue, allowing the laser to photo-activate it efficiently at the tissue interface. It may be speculated that the RB ability of producing singlet oxygen, upon light irradiation, may play a role in crosslinking tissue collagen and chitosan via their amino groups[7,8]. A recent study on the rose adhesive showed also that the maximum temperature achieved during the tissue-adhesive bonding was ~32 °C, supporting the hypothesis of a photochemical process[4]. Other sutureless techniques for tissue repair are currently investigated by several research groups. Laser tissue welding (LTW) for example has been successfully applied in ophthalmic surgery by Pini *et al* [9]. In general though, LTW can cause collateral thermal damage as tissue temperature can reach 65-75 °C during laser irradiation. Cyanoacrylate glues are also applied clinically to close skin wounds instead of sutures; nevertheless they are generally not used for internal organ repair due to their toxicity [10]. Although the rose adhesive (~15 KPa) has higher bonding strength than fibrin glues (~8 kPa) [11], cyanoacrylate glues still provide the strongest adhesion (~150 kPa) [12]. The thickness of the rose adhesive requires particular attention during fabrication: a thick adhesive (≥20 µm) would, for example, prevent the laser from reaching the tissue interface and would weaken the bonding strength due to excessive absorption of the laser by the rose bengal. A thin adhesive (<10 µm), on the other hand, would increase the laser irradiance and fluence at the adhesive-tissue interface. However, care should be taken in reducing the film thickness in order to prevent excessive heating of tissue during laser irradiation. The rose adhesive induces no significant cytotoxic effects on human fibroblasts[4] and has thus a promising use in repairing soft tissue inside the body, such as intestine and peripheral nerves. This adhesive can also have applications in tissue engineering: It can be integrated, for example, in a bandage with extracellular matrices to repair tissue and enhance wound healing without the aid of sutures[13]. It should be noted that we have not observed any significant adverse effects, including localized inflammation, to be associated with this procedure.

Disclosures

No conflicts of interest declared.

Acknowledgements

This work was supported by the UWS Research Grant.

References

1. O'Neill, A.C., Winograd, J.M., *et al.* Microvascular anastomosis using a photochemical tissue bonding technique. *Lasers Surg. Med.* **39**, 716-22 (2007).
2. Yao, M., Yaroslavsky, A., *et al.* Phototoxicity is not associated with photochemical tissue bonding of skin. *Lasers Surg. Med.* **42**, 123-31 (2010).
3. Lauto, A., Mawad, D., *et al.* Photochemical Tissue Bonding with Chitosan Adhesive Films. *Biomedical Engineering OnLine.* **9**, 47 (2010).
4. Chan, B.P., Kochevar, I.E., *et al.* Enhancement of porcine skin graft adherence using a light-activated process. *J. Surg. Res.* **108**, 77-84 (2002).
5. Lauto, A., Faster L.J., *et al.* Sutureless nerve repair with laser-activated chitosan adhesive: a pilot *in vivo* study. *Photomed. Laser Surg.* **26**, 227-34 (2008).
6. Lauto, A., Hook, J., *et al.* Chitosan adhesive for laser tissue repair: *in vitro* characterization. *Lasers Surg. Med.* **36**, 193-201 (2005).
7. Au, V., Madison, S.A., *et al.* Effects of singlet oxygen on the extracellular matrix protein collagen: oxidation of the collagen crosslink histidinohydroxylysinonorleucine and histidine. *Arch. Biochem. Biophys.* **384**, 133-42 (2000).
8. Encinas, M.V., Rufs, A.M., *et al.* Xanthene dyes/amine as photoinitiators of radical polymerization: A comparative and photochemical study in aqueous medium. *Polymer.* **13**, 2762-2767 (2009).
9. Pini, R., Rossi, F., *et al.* A new technique for the closure of the lens capsule by laser welding. *Ophtalmic Surgery Lasers & Imaging.* **39**(3), 260-26 (2008).
10. Wieken, K., Angioi-Duprez, K., & Lim, A. Nerve anastomosis with glue: comparative histologic study of fibrin and cyanoacrylate glue. *J. Reconstr. Microsurg.* **19**, 17-20 (2003).
11. Menovsky, T. & Beek, J.F. Laser, fibrin glue, or suture repair of peripheral nerves: a comparative functional, histological, and morphometric study in the rat sciatic nerve. *J. Neurosurg.* **95**, 694-9 (2001).
12. García, P., Jorge H.E., *et al.* Comparative study of the mechanical behavior of a cyanoacrylate and a bioadhesive. *J. Mater. Sci. Mater. Med.* **15**(2), 109-15 (2004).
13. Lauto, A. Integration of extracellular matrix with chitosan adhesive film for sutureless tissue fixation. *Lasers Surg. Med.* **41**, 366-71 (2009).

