TISSUE ENGINEERING: Part A
Volume 23, Numbers 3 and 4, 2017

DOI: 10.1089/ten.tea.2016.0266

termis
Tissue Engineering
& Regenerative Medicine
International Society

ORIGINAL ARTICLE

Direct Bonding of Chitosan Biomaterials to Tissues Using Transglutaminase for Surgical Repair or Device Implantation

Javier G. Fernandez, PhD,[1,2,*] Suneil Seetharam, MSc,[1] Christopher Ding, MSc,[1] Juani Feliz, MSc,[1] Ed Doherty, PhD,[1] and Donald E. Ingber, MD, PhD[1–4]

Natural biomaterials, such as chitosan and collagen, are useful for biomedical applications because they are biocompatible, mechanically robust, and biodegradable, but it is difficult to rapidly and tightly bond them to living tissues. In this study, we demonstrate that the microbial transglutaminase (mTG), can be used to rapidly (<5 min) bond chitosan and collagen biomaterials to the surfaces of hepatic, cardiac, and dermal tissues, as well as to functionalized polydimethylsiloxane (PDMS) materials that are used in medical products. The mTG-bonded chitosan patches effectively sealed intestinal perforations, and a newly developed two-component mTG-bonded chitosan spray effectively repaired ruptures in a breathing lung when tested *ex vivo*. The mechanical strength of mTG-catalyzed chitosan adhesive bonds were comparable to those generated by commonly used surgical glues. These results suggest that mTG preparations may be broadly employed to bond various types of organic materials, including polysaccharides, proteins, and functionalized inorganic polymers to living tissues, which may open new avenues for biomedical engineering, medical device integration, and tissue repair.

Keywords: biomimetic materials, biomedical engineering, chitosan

Introduction

CHITINOUS BIOLOGICAL STRUCTURES, such as those found in insect cuticles and crustacean shells, have inspired the development of numerous biomedical materials due to their extraordinary strength and toughness.[1] For commercial applications, chitin is industrially extracted from shrimp shells and deacetylated to the more technologically relevant chitosan that can be used to produce three-dimensional shapes.[2] Chitosan consists of a cellulose backbone with an amine group introduced at the C-2 position of 75–90% of its individual glucose units, whereas the remaining glucose units retain the original acetyl group. This combination of a strong cellulose backbone, a high concentration of highly reactive free amine groups, and the ability to be molded, makes chitosan an ideal polymer for medical applications.

However, it is still difficult to quickly and strongly bond chitosan materials to living tissues. Failure to form this type of stable bond restricts its use for certain clinical applications. For example, quick establishment of a strong material–biological tissue interface is critical for wound repair, sealing of organ perforations, and protection of large areas of tissue damage, particularly in emergency and casualty evacuation. Micromovements between medical implants relative to patient tissues are also a common cause of implant rejection,[3] and the stability of the physical interface between electrochemical biosensors and the biological tissues they monitor is crucial for the accuracy of medical diagnosis and real-time monitoring of physiological parameters.[4]

Thus, in the present study, we set out to develop a method to rapidly and tightly adhere chitosan biomaterials to biological tissues to open up new paths for biomedical engineering and medical device development. We focused on the use of transglutaminase (TG) enzyme because it provides a rapid and highly efficient way to bond primary amine groups, which are found at high density in chitosan and other biological molecules, such as collagen, which are

[1]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts.
[2]Harvard School of Engineering and Applied Sciences, Harvard University, Cambridge, Massachusetts.
[3]Vascular Biology Program, Department of Surgery, Children's Hospital and Harvard Medical School, Boston, Massachusetts.
[4]Vascular Biology Program, Department of Pathology, Children's Hospital and Harvard Medical School, Boston, Massachusetts.
Current affiliation: Singapore University of Technology and Design, Singapore.

relevant to tissue engineering and regenerative medicine. Tissue-derived TG has been employed to bond pieces of cartilage[5] and microbial TG (mTG) has been used to crosslink protein gels, including fibrin and gelatin gels, which were shown to exhibit improved cell attachment and resistance to protease degradation.[6] TGs also have been investigated for the crosslinking of proteins to nonproteinaceous molecules that the required functional groups, such as hyaluronic acid[7] and peptide-modified polyethyleneglycol (PEG).[8]

We chose to use mTG rather than tissue-derived TG in the present study because it can be produced much more efficiently at a lower cost, and it does not require the presence of calcium ions to be activated, which enables many applications not supported by tissue-derived TG. Our studies show that mTG can be used to bond chitosan and collagen materials to organic substrates, including different tissue types as well as inorganic substrates modified to contain sterically available amine groups, but this process is only generalizable when the mTG preparation contains casein, which is rich in glutamic acid residues. We also show that mTG-mediated bonding of films made of chitosan strengthened with fibroin can be used to effectively seal intestinal perforations and a specifically designed mTG-based chitosan spray can be used to repair an injured breathing lung using *ex vivo* models.

Results and Discussion

mTG preparations with casein efficiently bond chitosan to organic surfaces

When glutamine residues are exposed to mTG in the absence of a contacting substrate containing primary amines, the enzyme catalyzes the hydrolysis of the residues, resulting in the loss of the material's amine groups (Fig. 1a). However, when another structure is present containing primary amines (e.g., chitosan), mTG catalyzes an acyl transfer reaction between a glutaminyl residue (acyl donor) and a primary amine (acyl receptor),[9] thereby covalently linking to the structure. This process is extremely fast and gives rise to strong covalent bonds with high resistance to degradation.[10]

In common mTG preparations, carbohydrates (e.g., maltodextrin, saccharose, or mannose) are added to increase the stability of the enzyme against thermal degradation,[11] and proteins, such as casein, are added to protect mTG against degradation by extracellular proteolytic enzymes.[12] Thus, to investigate mTG as a bonding reagent for chitosan materials, we first compared the ability of two different commercial mTG preparations to bind chitosan and collagen films: one preparation contained only maltodextrin (mTG+Ma) and another contained both this carbohydrate and casein

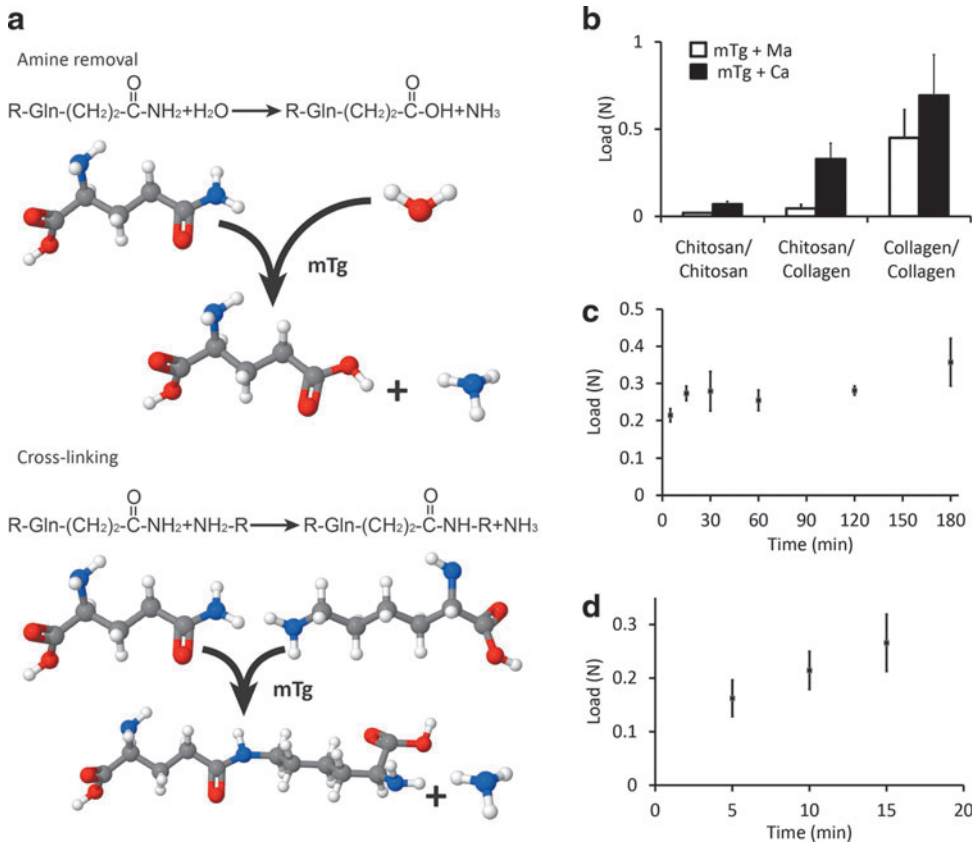

FIG. 1. The mTG reaction and mTG-mediated bonding of biomaterial films. **(a)** mTG-catalyzed reactions with relevance for biomaterial bonding. The upper reaction is a deamination that occurs in the absence of amine substrates and the presence of water. mTG catalyzes the hydrolysis of the glutaminyl residue, resulting in loss of the amine group. In the lower reaction, the presence of a primary amine group results in the formation of a covalent bond between both molecules. The involved atoms are O (*red*), N (*blue*), and H (*white*). **(b)** Bonding strengths produced by commercial mTG preparations containing mTG and maltodextrin (mTG+Ma) or both components plus casein (mTG+Ca). **(c)** Kinetics of the mTG-bonding reaction measured at the macroscale as the adhesive force between surfaces of two muscle tissues. **(d)** More detailed examination of the first 15 min of the reaction dynamics shown in **(c)**. mTG, microbial transglutaminase.

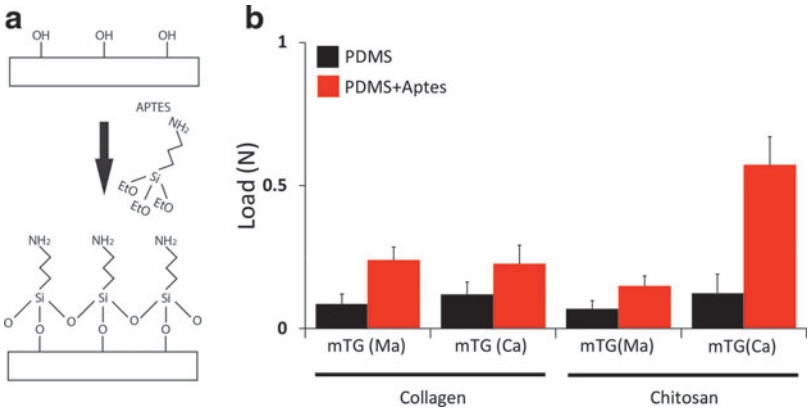

FIG. 2. mTG-mediated bonding of biomaterials to an inorganic PDMS substrate. **(a)** Chemistry that underlies APTES functionalization of the PDMS surface. The highly reactive silane group in APTES silanizes the surface by forming covalent bonds with surface atoms. **(b)** Bonding strength produced between contacting chitosan or collagen films and untreated (PDMS) or APTES-modified PDMS (PDMS+Aptes) substrates. PDMS, polydimethylsiloxane.

(mTG+Ca). The mTG preparation was applied as a powder to one of the surfaces before the two materials were brought together without applying pressure; because both surfaces are hydrated and hydrophilic, capillary forces held the films in close contact. When we quantified the adhesion force required to separate two mTG-bonded films 10 min after mTG application using a standard t-peel test protocol (ASTM D1876), we found that while the mTG+Ma preparation effectively bonded collagen substrates together, it was unable to bond chitosan substrates to each other or to collagen surfaces.

On the other hand, the preparation containing casein (mTG+Ca) bonded collagen to both itself and to chitosan substrates, although it still did not mediate chitosan–chitosan bonding (Fig. 1b). Thus, the presence of casein (which is rich in glutamic acid residues) in the preparation appears to be necessary for mTG to be able to rapidly and tightly bind chitosan to other organic surfaces, such as collagen, however is not necessary to mediate the collagen–collagen bonding, as the collagen protein already contains many internal glutamic acid residues. This finding that different mTG preparations produce different bonding results might also explain contradictions in past rheological studies of biopolymeric gels formed by mTG crosslinking, where identities of mTG stabilizers were not reported.[13–15]

Importantly, while the optimal reaction temperature for material bonding by mTG has been reported to be around 50°C, we observed rapid bonding of two organic (i.e., collagen) films even at room temperature using our mTG+Ca preparation (Fig. 1c). Moreover, the maximum load necessary to separate these films was reached within 15 min at room temperature (Fig. 1c, d), with more than 50% of the maximum resistance to separation already being reached within 5 min after the reaction was initiated (Fig. 1d). Thus, the mTG preparation with casein enables rapid and strong bonding of chitosan and collagen materials to organic surfaces, indicating its suitability for use under physiological conditions in medically relevant tissue microenvironments.

mTG-mediated bonding of chitosan and collagen to inorganic materials

Implanted medical devices, electrochemical sensors, and actuators require strong and preferentially seamless interfaces between inorganic materials and living tissues, as do recently developed microfluidic ''organ-on-a-chip'' culture devices.[16,17] Silicone is an example of a material that has been extensively used in medical technologies since it was first approved by the FDA for use as a material for surgical reconstruction after mastectomy and correction of congenital deformities because of its high level of tear resistance. Polydimethylsiloxane (PDMS) is a specific type of silicone polymer broadly used in medical devices, as well as microfluidic organ-on-a-chip devices, because it can exhibit a broad range of different mechanical properties based on subtle variations in the number of monomers and degree of crosslinking, as well as inclusion of additives. However, silicone materials do not normally serve as substrates for mTG-mediated adhesion because they lack endogenous reactive amine groups.

We explored if we could expand mTG catalyzed adhesion of engineered organic biofilms to include bonding of these biofilms to PDMS by first functionalizing its surface with amine groups using 3-Triethoxysilylpropylamine (APTES) before addition of mTG (Fig. 2a). We then carried out similar bonding studies using collagen or chitosan films with mTG+Ma versus mTG+Ca, and measured adhesion strength. Both collagen and chitosan films show limited adhesion to unfunctionalized PDMS, without any significant differences between the material and preparation used (p-values ranged between 0.2 and 0.9) (Fig. 2b). In contrast, pretreatment with APTES resulted in a significant increase of bonding strength between films of chitosan or collagen and PDMS surfaces using mTG+Ca ($p = 0.007$ and $p = 0.04$, respectively), with the chitosan–PDMS bond displaying greater strength of adhesion (Fig. 2b).

There was no detectable difference in the strength of bonding between collagen films and the functionalized PDMS surfaces using the different mTG preparations (i.e., mTG+Ma vs. mTG+Ca, $p = 0.8$). This is in direct contrast to the results with chitosan films, where only the casein-containing mTG preparation was able to effectively bond the chitosan to the functionalized PDMS ($p = 0.006$), and there was no significant effect on bonding when mTG+Ma was used with functionalized or unfunctionalized PDMS ($p > 0.051$).

Together, these findings suggest that mTG preparations containing casein can mediate bonding of chitosan and collagen to inorganic surfaces, such as PDMS, which are integral parts of various types of biomedical implants and microfluidic devices. This approach could facilitate the development of medical technologies with synthetic elements, such as sensors, actuators, or structural components, and allow them to leverage the biocompatibility and absorbability of these natural biomaterials.

Comparison of mTG-mediated bonding versus surgical glues

To investigate the potential of mTG as a medical bonding reagent for natural biomaterials, we compared the mechanical

strength of a chitosan patch bonded to a collagen film using the mTG+Ca preparation versus the bonding strength produced using two common commercial surgical glues, Evicel and Progel. Evicel (Johnson & Johnson) is fibrin based and the most broadly employed sealant for surgical applications. Evicel includes two separate components that are applied and combined simultaneously with a double-barrel syringe, which results in their crosslinking and production of a sealant layer that is primarily used as a mechanical reinforcement for surgical sutures and staples. Progel (C.R. Bard) is an example of a newer generation of surgical glues specifically used for the treatment of pleural air leaks that are based on the use of a functional polymer and a bonding reagent (in the case of Progel, PEG functionalized with succinate groups and human serum albumin, respectively). Similar to Evicel, Progel comes as two components that are applied simultaneously; however, crosslinking of Progel is much more rapid (i.e., it exists in a liquid form for only a very short time) and, hence it stiffens more quickly and is less conformable to the applied tissue.

We assayed all three materials with the "*Standard Test Method for Burst Strength of Surgical Sealants*" (ASTM F2392), a standard industrial protocol that measures the ultimate pressure (pressure necessary to break the sealing) of a surgical patch covering a 3 mm circular perforation on standard tissue mimic surface (e.g., collagen film #320; Nippi, Inc., Japan) (Fig. 3a). As per the ASTM protocol, the ultimate pressure is reported as the average pressure ($n=5$) necessary to break the sealing. However, due to variability introduced during the application, we also report maximum pressure reached among all the samples, as it is indicative of bonding strength produced by each adhesion method independently of the user's skills.

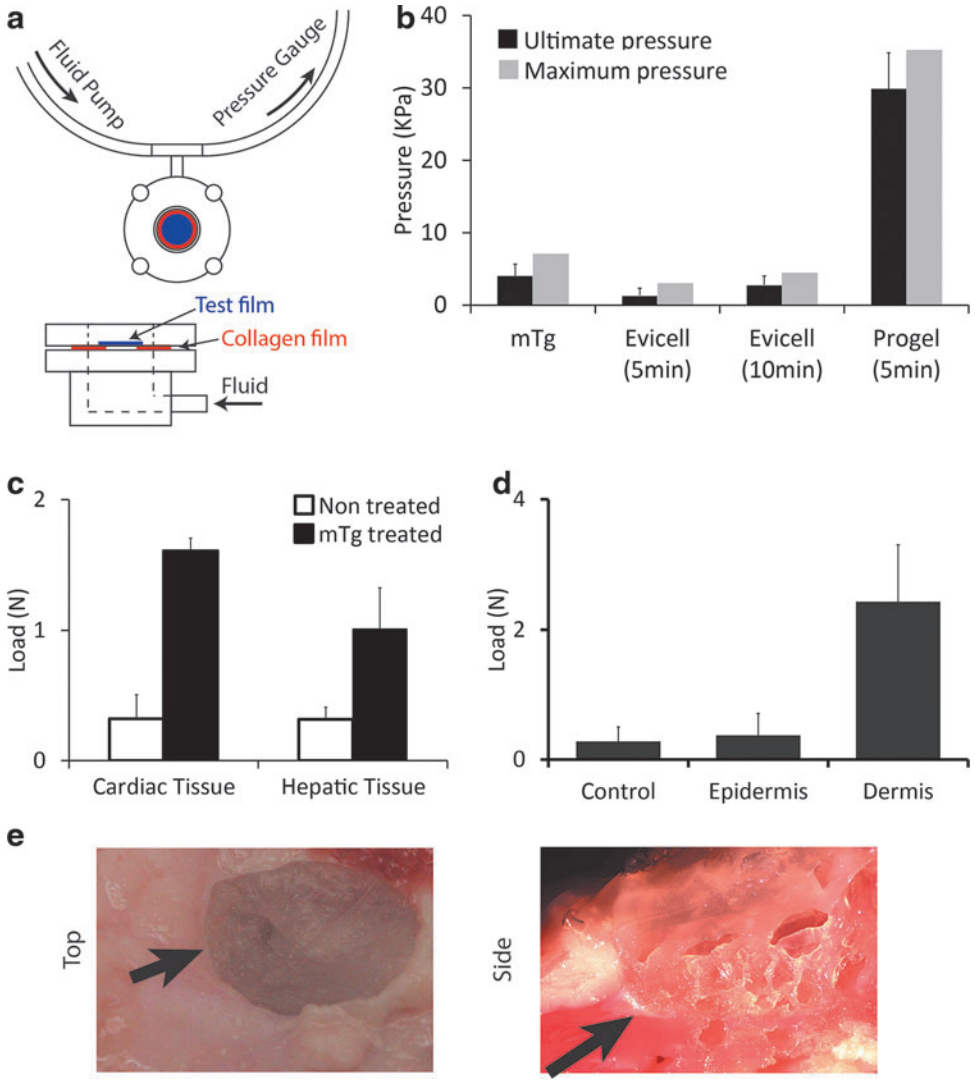

FIG. 3. Applicability of mTG bonding of biomaterial films and foams to native tissues. **(a)** Diagram of the ASTM F2392 standard protocol to measure burst strength of surgical sealants that are used to fill a 3 mm diameter hole on a collagen surface. **(b)** Performance of different sealing materials tested in the ASTM F2392 assay presented as average maximum and ultimate pressures required to burst the seals. **(c)** Bonding strength of a chitosan film bonded to the heart and liver surface using mTG and casein (mTG+Ca) preparations. **(d)** Bonding strength of a chitosan film adhered to skin epidermis or dermis (after epidermis is removed); control indicates bonding of chitosan to dermis; however, no adhesion is observed to undamaged (fully cornified) epidermis. **(e)** Photomicrograph of a chitosan foam bonded to the surfaces of a 1-cm irregularly shaped defect in an explanted pig muscle using mTG+Ca (note the seamless nature of the adhesion).

These studies revealed that although both glues produced clinically relevant bonding strengths, the Progel glue provided much stronger adhesive properties than the Evicel glue (Fig. 3b). However, the Progel material was less flexible and conformal than the Evicel glue, which produced clumps rather than conformal coatings (not shown). Importantly, although the mechanical adhesive strength of the mTG-mediated bond between the chitosan and collagen films was not as strong as that of the Progel, it was significantly greater than that of the Evicel ($p=0.02$) and thus, the mTG BioGlue produced a material bond that is well in the range of medically useful sealants. These findings suggest that mTG-mediated bonding of chitosan materials may be well suited for a spectrum of biomedical applications, where rapid and strong bonding of biomaterials is required.

mTG-mediated chitosan bonding to whole tissues

To investigate the medical potential of mTG-mediated bonding of chitosan to living tissues, we used this mTG+Ca method to attach chitosan films to whole porcine liver, heart, and skin explants. Chitosan films were covered with the mTG powder and placed on the surface of the tissue for 10 min at room temperature (Supplementary Fig. S1b; Supplementary Data are available online at www.liebertpub.com/tea). Again, no pressure was applied during the reaction time, and instead the film conformed to the shape and held itself in place because of the small amounts of water it holds on its surface and the action of resultant capillary forces.

These studies revealed that the addition of mTG resulted in a 3- to 6-fold increase in the strength of bonding between chitosan films and liver ($p=0.03$) or heart ($p=0.002$) tissues, respectively (Fig. 3c). In contrast, when we tried to use mTG to adhere chitosan to the epidermal surface of the skin explant, it did not produce any detectable ($p=0.7$) bonding of the film to the tissue (Fig. 3d). This is to be expected given that the stratum corneum of the epidermis is rich in endogenous tTG, which results in crosslinking of virtually all available amines within the epidermal cell layer,[18] and apparently, none is available to link to chitosan using exogenous mTG. This possibility was supported by the finding that mechanical removal of the epidermis and exposure of the underlying dermis resulted in extraordinarily strong bonding to chitosan films using mTG ($p=0.02$), even surpassing that observed for other organ surfaces (Fig. 3d).

We explored whether mTG also can be used to bond chitosan foams to tissues to help fill larger tissue defects. We confirmed that chitosan foams can be similarly bonded to tissues by using mTG+Ca to adhere chitosan foams to an irregularly shaped 1-cm defect in an explanted *latissimus dorsi* muscle from a domestic pig. Again, these foams exhibited firm attachment to the tissue boundaries within 10 min after application, and the adhesion between the foam and tissue was virtually seamless with the border being difficult to detect when analyzed with photomicroscopy (Fig. 3e and Supplementary Fig. S1a).

Finally, to evaluate the biocompatibility and host response to mTG-bonded chitosan foams *in vivo*, we used mTG to bond chitosan foams to the surface of the *Mus musculus* muscle subcutaneously in the lumbar area of a mouse, and then removed the materials 2 or 4 weeks later (Supplementary Fig. S2a). Importantly, we noted no apparent degradation of the foam material over 1 month, indicating that it is stable over time when bonded to living tissues. We detected a high level of cell and tissue penetration into the pores of the foam with 2 weeks, and fibrous granuloma deposits appeared surrounding the material after 4 weeks; however, the histological analysis of the foams showed little presence of foreign body-type giant cells (Supplementary Fig. S2b). These findings are consistent with earlier observations that demonstrated implanted chitosan materials stimulate the formation of granulation tissue, and produce minimal chronic inflammation or foreign body reaction with a relative absence of giant cells when implanted *in vivo*.[19–21]

mTG-bonded chitosan films as surgical sealants

To further explore the potential biomedical uses of mTG-bonded chitosan materials, we examined whether mTG-mediated bonding of chitosan films could quickly seal a punctured tissue. Gastrointestinal perforations occur in a variety of illnesses, including Crohn's disease, appendicitis, and ulcers, and if not treated in time, they lead to life-threatening abdominal infections and sepsis. Except for very mild cases, their repair requires a surgical procedure that reestablishes a tight barrier separating the gastrointestinal lumen and the abdominal space.

We developed an *ex vivo* intestinal perforation model by surgically incising a 1 cm slit hole in the wall of an explanted pig small intestine. To repair the perforation, we used mTG+Ca to bond a $3\times3\,cm^2$ chitosan film strengthened with fibroin (i.e., Shrilk[22]) over the hole by placing the mTG powder-coated film in contact with the inner surface of the intestinal wall surrounding the damaged area (e.g., mimicking placement through endoscopic surgery). We then carried out a burst test 10 min later by clamping both ends of each intestinal segment onto the end of a hollow tube and flowing saline into the lumen to increase intraluminal hydraulic pressure. Impressively, the chitosan–fibroin composite film seal systematically remained intact even when the pressure was raised to more than 125 kPa (Fig. 4a); however, the experiment ended when the intestinal wall opposite to the site of the sealed perforation burst (Fig. 4b and Supplementary Videos S1 and S2). The intestine burst at pressure values 20 times higher than the physiological pressure present in the intestine[23]; this pressure is even higher than levels in the circulatory system experienced during a hypertensive crisis, which is the highest physiological pressure generated within the human body that we have found in the literature. Thus, chitosan patches sealed in place using mTG could potentially be used for surgical repair for various tissues.

A sprayable mTG-bonded method for repair of lung punctures

Although we used chitosan films to seal punctures in the intestine, the organ was explanted and static in that study. It would be more difficult to use a film of fixed shape and size to seal punctures and tears in living organs that experience dynamic movements, such as in the lung that undergoes cyclic breathing motions, or in organs with irregular-shaped defects. We, therefore, explored whether we could develop a sprayable form of chitosan and mTG sealant. One major challenge that we had to overcome was that chitosan and casein exhibit opposite behaviors in basic and acidic environments; chitosan dissolves in acid and precipitates in alkaline solutions, whereas the opposite is true for casein.

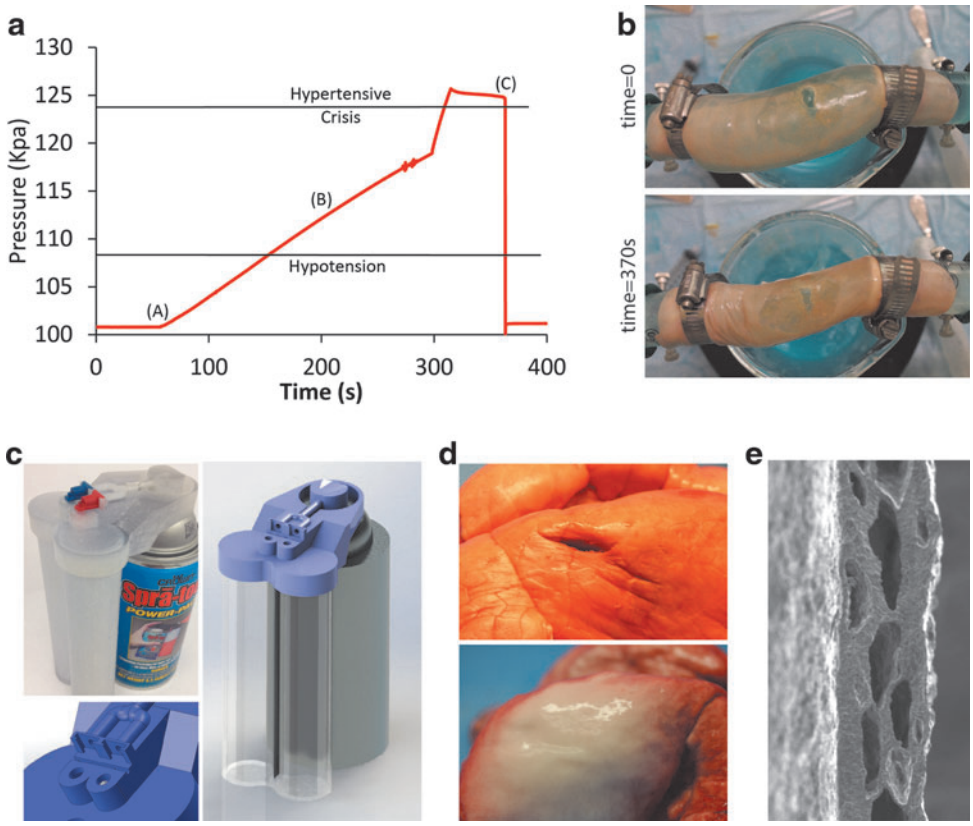

FIG. 4. Use of mTG as a sealant for tissue punctures. **(a)** An example of a burst test performed on a 1 cm diameter hole in the wall of an explanted pig small intestine, repaired with a chitosan film patch bonded to the tissue with mTG+Ca. The patch resisted a linear increment of hydraulic pressure applied through the intestinal lumen for several minutes, reaching the equivalent to the maximum physiological pressures in the human body before bursting. **(b)** Images of the chitosan patch before (*top*) and after (*bottom*) a region of the native intestine on the opposite side from the patch burst at high pressure, which corresponds to point C on the graph shown in **(a)** (Supplementary Video S1). **(c)** Image and digital rendering of the double-canister spray device designed to form a conformal coating of chitosan with mTG+Ca with adhesive properties. Each component is loaded into separate barrels, and pushed through the nozzles using the same pressure source. The exchangeable nozzles (*blue* and *red* in the image) have different output diameters, enabling control of the ratio between different components in the mix. **(d)** Photographs of a 3-cm deep puncture in an explanted pig lung before (*top*) and after (*bottom*) it was sprayed for 5 min while cyclically inflating and deflating the lungs using the double-canister spray method (Supplementary Video S3). **(e)** A scanning electron microscopic image of the chitosan+mTG+CA film produced at the surface of the lung using the spray method, which demonstrates its highly porous nature.

Thus, to create a sprayable form of chitosan, it must be dissolved in a diluted acid, and this prevents the combination of both components in a single spraying solution.

To overcome the different pH dependencies of chitosan and casein, we developed a double-canister spray system (Fig. 4c) in which one canister contained chitosan in an acidic solution and the other contained mTG with casein in a lightly basic solution, but then both solutions are sprayed simultaneously onto the same surface. When both components interact at the tissue surface, the pH of the combined solution neutralizes and gives rise to an even and homogeneous chitosan–mTG–casein precipitate with adhesive properties. The resulting properties of the conformal coating produced by the two-component spray differed from those of pure chitosan films that we used to test bonding strength in that they are much more elastic, but less tough.

We then investigated this double-spray system's ability to seal a 3×1 cm deep incision that was made on the surface of one lobe of an explanted pig lung, whereas the damaged lung was cyclically inflated and deflated to reproduce the breathing cycle using an external air source connected to the trachea (Supplementary Videos S3 and S4). By using the double-canister spray system to apply chitosan and mTG+Ca directly to the injured surface of the breathing lung, we were able to cover the puncture and the surrounding area with a conformal elastic film that effectively sealed the leak within a few minutes (Fig. 4d and Supplementary Fig. S1c). Scanning electron microscopic analysis of the sealed tissue surface revealed a seamless bond, as well as the porous nature of the sprayed chitosan+mTG+Ca sealant (Fig. 4e). The speed and easy applicability of the double-spray chitosan-mTG sealant method, combined with the biocompatibility and antimicrobial properties of chitosan, should make it useful for the treatment of internal and external wounds of variable sizes, including exposed dermal surfaces of burn injuries. Interestingly, based on our finding that mTG does not bond chitosan to epidermis, this method potentially can be used to create a sprayable skin for burn injuries that could serve as a protective cuticle that spontaneously detaches over time as the epidermal cells migrate into the healing wound.

Although we did not study these bonded materials *in vivo*, past studies have shown that chitosan implants only induce minimal chronic inflammation and foreign body reaction *in vivo*.[21] Together with their favorable mechanical adhesion properties, this makes mTG-mediated bonding of chitosan materials an ideal method to stably bond functional chitosan implants in different tissue contexts. Bonding of porous chitosan foams to dermis, for example, might be useful for mechanical treatment strategies used for large nonhealing wounds, such as Vacuum-Assisted Closure therapy, which would benefit from the use of bioabsorbable porous scaffolds that provide both good physical properties and strong attachment to the wound site.[24]

In conclusion, we have demonstrated how mTG preparations containing casein can be used to bond chitosan to materials composed of proteins, such as collagen and living tissues, as well as to inorganic surfaces of polymers that are used in medical and microfluidic devices, such as PDMS. The rapid action and the versatility of the bonding process make it suitable for a broad number of applications, ranging from surgical sealants to coatings for implantable medical devices or sensor/actuators to better integrate biomaterials and living tissues within microfluidic devices or BioMEMS. In short, the ability of mTG to rapidly and strongly bond chitosan and other biomaterials to both living tissues and inorganic surfaces offers a new way to seamlessly integrate living and nonliving materials.

Materials and Methods

Transglutaminase preparations

Two microbial transglutaminase (mTG) preparations containing Streptoverticillium calcium-independent TG were obtained from Ajinomoto Food Ingredients LLC (Chicago). One preparation contained ~1% (w/w) enzyme stabilized in maltodextrin, whereas the other was stabilized using maltodextrin (0.39 w/w) and casein (0.6 w/w). Enzymatic preparations were stored in a powder form to facilitate transport of mTG at room and higher temperatures, and because this formulation could enable its use across a broad spectrum of applications in the future (e.g., first aid and battlefield assistance). In the case of films and foams, we employed the enzymatic preparations directly in their powder, sprinkling the surface of the material with an even thin coating before apposing it to another surface for attachment. Unfortunately, as we applied the mTG as a powder, it is not possible to quantify the efficiency of the reaction. However, we could visually detect when the reaction went to completion because the mTG powder has a white color that fades away as the reaction moves to completion (Supplementary Fig. S1b), whereas the white powder remains visible if excess enzyme is applied. Thus, we were able to empirically estimate the amount of mTG required for crosslinking by providing the maximum amount of mTG that would produce optimal mechanical strength without producing residual white powder remnants. In the case of the double-canister spray system, mTG preparations were used as a 3% (w/v) solution in 4% (w/v) NaOH to maintain their activity.[9] Although higher mTG concentrations enhanced the film-forming capabilities of the spray components, they were not useful for spraying due to micelle formation in the solution.

Chitosan and collagen materials

Chitosan films and foams were produced as reported before.[1,19] We used chitosan films to investigate bonding strength using standardized industrial methods and ASTM protocols; chitosan foams were used to investigate tissue defect filling. Chitosan films were produced by solvent evaporation casting of a 2% (w/v) solution of 80% deacetylated chitosan in a 1% (v/v) acetic acid solution. Films were neutralized with a 4% NaOH solution (w/v). Chitosan foams were made by freeze drying a 1% (w/v) solution of chitosan in a 0.5% (v/v) acetic acid solution, neutralizing it in 4% (w/v) NaOH, and intensely washing in doubly ionized water. Due to the randomness of the foam structure, this configuration is not suitable for a standardized measurement of bonding strength, and we carried out studies with living tissues instead, as described in the Results and Discussion section. Collagen films were produced from the Collagen casing No. 320 (Nippi, Inc., Japan) standard for ASTM protocols (e.g., ASTM F2392). Chitosan films strengthened with fibroin (i.e., Shrilk) were produced by sequential deposition and neutralization of the components, as described previously.[22]

APTES deposition on PDMS surfaces was produced in aqueous phase. The PDMS samples were washed and dried before being treated with oxygen plasma for 30 s. The action of the plasma causes three main processes to occur: cleaning, ablation, and chemical modification. The cleaning causes any organic surface contamination to be removed from the polymer, preventing its interference in the chemical modification processes. Chemical modification involves the production of new surface hydroxyl groups available to interact with the APTES solution. Immediately after plasma treatment, PDMS samples were immersed in APTES solution in deionized water (1% v/v) for 20 min and immediately washed with de-ionized water to remove excess of un-crosslinked APTES.

Mechanical adhesion testing

Adhesion measurements were performed using two ASTM methods. ASTM D1876 (t-peel test) experiments were performed with an Instron 3342 instrument (500N; Instron) to measure the strength of adhesion of films bonded to flat surfaces (Supplementary Fig. S1d). Studies were carried out on biopolymer films, collagen substrates, and tissues (i.e., skin, lung, heart, and muscle) shaped in rectangular (1 cm wide × 6 cm long) strips. Both surfaces of the adhesion test were fixed to an aluminum support (200 μm thick) to avoid the effect of the film and substrate stretching when measuring surface adhesion; pulling speed was 10 mm/min. The treated side of the films was placed in direct contact, but they were not subjected to pressure during the reaction time, and instead were held in place by capillary forces.

To measure burst pressures, we used the ASTM F2392 standard protocol, which was developed to measure burst strength of surgical sealants. A 4 mm diameter patch of chitosan and mTG+Ca powder was used to cover a 3 mm diameter hole punched in the center of a collagen film (Collagen casing No. 320; Nippi, Inc., Japan) prepared as per the ASTM protocol. Assays were performed 5 min after application of the adhesive (mTG or surgical sealant) in all studies. The system was filled with water on top of the patch and air was delivered with a syringe pump (World Precision Instruments, Inc., Florida) at a rate of 2 mL/min from the bottom. Pressure was measured using a differential pressure sensor (Pasco, CA). Burst pressure was observed as a drop in pressure, whereas air bubbles were observed passing through the broken seal of the patch. In all the mechanical tests, the sample size and

statistical significance were chosen assuming a normal distribution,[25] and all error bars indicate standard deviation.

Lung tests with the double spray were performed using explanted lungs of domestic pig (*sus domesticus*) obtained from a local slaughterhouse. A deep hole (3×1.5 cm) was formed on the surface of one lung, and the trachea was connected to an external air pump. Continuous cyclic airflow to fully inflate and deflate the lungs was provided to mimic breathing motions of the lung. Tests were carried out twice on the same pair of lungs. Intestinal tests were performed by attaching both ends of a 15 cm section of explanted small intestine from a domestic pig to 3 cm diameter tube. A 1 cm hole was incised in the intestine wall and subsequently repaired using mTG in powder form to attach a 3-cm chitosan–fibroin (i.e., Shrilk) patch from inside the lumen of the intestine. The system was filled with pressurized water containing blue dye (to enhance contrast) and pressure was measured using a differential pressure sensor (Pasco, CA).

Acknowledgments

This work was supported by the Wyss Institute for Biologically Inspired Engineering at Harvard University. The authors would like to thank Benjamin Boettner for his useful comments and support.

Authors' Contributions

J.G.F. and D.E.I. conceived the project, J.G.F. and S.S designed and performed spray test on lungs, J.G.F. and D.E.I. fabricated the double-spray system, J.G.F., J.F., and E.D. designed and performed ASTM experiments, J.G.F. and D.E.I. analyzed the results and wrote the article.

Disclosure Statement

No competing financial interests exist

References

1. Fernandez, J.G., and Ingber, D.E. Bioinspired chitinous material solutions for environmental sustainability and medicine. Adv Funct Mat **23,** 4454, 2013.
2. Fernandez, J.G., and Ingber, D.E. Manufacturing of large-scale functional objects using biodegradable chitosan bioplastic. Macromol Mater Eng **299,** 932, 2014.
3. Freitas, Jr., A.C., *et al.* The effect of implant design on insertion torque and immediate micromotion. Clin Oral Implants Res **23,** 113, 2012.
4. Zhou, M., and Dong, S. Bioelectrochemical interface engineering: Toward the fabrication of electrochemical biosensors, biofuel cells, and self-powered logic biosensors. Acc Chem Res **44,** 1232, 2011.
5. Jürgensen, K., *et al.* A new biological glue for cartilage-cartilage interfaces: tissue transglutaminase. J Bone Joint Surg Am **79,** 185, 1997.
6. Chau, D.Y.S., *et al.* The cellular response to transglutaminase-cross-linked collagen. Biomaterials **26,** 6518, 2005.
7. Picard, J., Giraudier, S., and Larreta-Garde, V. Controlled remodeling of a protein-polysaccharide mixed gel: examples of gelatin-hyaluronic acid mixtures. Soft Matter **5,** 4198, 2009.
8. Sperinde, J.J., and Griffith, L.G. Control and prediction of gelation kinetics in enzymatically cross-linked poly(ethylene glycol) hydrogels. Macromolecules **33,** 5476, 2000.
9. Yokoyama, K., Nio, N., and Kikuchi, Y. Properties and applications of microbial transglutaminase. Appl Microbiol Biotechnol **64,** 447, 2004.
10. Lorand, L., and Graham, R.M. Transglutaminases: cross-linking enzymes with pleiotropic functions. Nat Rev Mol Cell Biol **4,** 140, 2003.
11. Cui, L., *et al.* Stabilization of a new microbial transglutaminase from Streptomyces hygroscopicus WSH03-13 by spray drying. Process Biochem **41,** 1427, 2006.
12. Junqua, M., *et al.* Optimization of microbial transglutaminase production using experimental designs. Appl Microbiol Biotechnol **48,** 730, 1997.
13. Schorsch, C., *et al.* Cross-linking casein micelles by a microbial transglutaminase conditions for formation of transglutaminase-induced gels. Int Dairy J **10,** 519, 2000.
14. Chen, T., *et al.* Enzyme-catalyzed gel formation of gelatin and chitosan: potential for in situ applications. Biomaterials **24,** 2831, 2003.
15. Benjakul, S., *et al.* Effect of chitin and chitosan on gelling properties of surimi from barred garfish (*Hemiramphus far*). J Sci Food Agric **81,** 102, 2001.
16. Huh, D., *et al.* Reconstituting organ-level lung functions on a chip. Science **328,** 1662, 2010.
17. Bhatia, S.N., and Ingber D.E. Microfluidic organs-on-chips. Nat Biotech **32,** 760, 2014.
18. Eckhart, L., *et al.* Cell death by cornification. Biochim Biophys Acta **1833,** 3471, 2013.
19. Fernandes, J.C., *et al.* Anti-inflammatory activity of chitooligosaccharides in vivo. Mar Drugs **8,** 1763, 2010.
20. Qiao, Y., Bai X.F., and Du, Y.G. Chitosan oligosaccharides protect mice from LPS challenge by attenuation of inflammation and oxidative stress. Int Immunopharmacol **11,** 121, 2011.
21. Bavariya, A.J., *et al.* Evaluation of biocompatibility and degradation of chitosan nanofiber membrane crosslinked with genipin. J Biomed Mater Res B Appl Biomater **102,** 1084, 2014.
22. Fernandez, J.G., and Ingber, D.E. Unexpected strength and toughness in chitosan-fibroin laminates inspired by insect cuticle. Adv Mater **24,** 480, 2012.
23. Tasaka, K., and Farrar, J.T. Intraluminal pressure of the small intestine of the unanesthetized dog. Pflugers Arch **364,** 35, 1976.
24. Nather, A., *et al.* Effectiveness of vacuum-assisted closure (VAC) therapy in the healing of chronic diabetic foot ulcers. Ann Acad Med Singapore **39,** 353, 2010.
25. Hess, P.E., *et al.* Uncertainties in material and geometric strength and load variables. Naval Eng J **114,** 139, 2002.

Address correspondence to:
Donald E. Ingber, MD, PhD
Wyss Institute for Biologically Inspired Engineering
3 Blackfan Circle
CLSB 5th floor
Boston, MA 02115

E-mail: don.ingber@wyss.harvard.edu

Received: July 1, 2016
Accepted: October 18, 2016
Online Publication Date: December 19, 2016