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Biocompatibility of adhesive complex coacervates modeled after the sandcastle glue of *Phragmatopoma californica* for craniofacial reconstruction

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ABSTRACT

Craniofacial reconstruction would benefit from a degradable adhesive capable of holding bone fragments in three-dimensional alignment and gradually being replaced by new bone without loss of alignment or volume changes. Modeled after a natural adhesive secreted by the sandcastle worm, we studied the biocompatibility of adhesive complex coacervates *in vitro* and *in vivo* with two different rat calvarial models. We found that the adhesive was non-cytotoxic and supported the attachment, spreading, and migration of a commonly used osteoblastic cell line over the course of several days. In animal studies we found that the adhesive was capable of maintaining three-dimensional bone alignment in freely moving rats over a 12 week indwelling period. Histological evidence indicated that the adhesive was gradually resorbed and replaced by new bone that became lamellar across the defect without loss of alignment, changes in volume, or changes in the adjacent uninjured bone. The presence of inflammatory cells was consistent with what has been reported with other craniofacial fixation methods including metal plates, screws, tacks, calcium phosphate cements and cyanoacrylate adhesives. Collectively, the results suggest that the new bioadhesive formulation is degradable, osteoconductive and appears suitable for use in the reconstruction of craniofacial fractures.

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1. Introduction

Traumatic injury to the face and skull can create many bone fragments that are difficult to accurately reposition with pins, plates, and screws [1,2]. In some cases due to the fixation technology in use small bone fragments may be discarded [3–5]. Precise alignment and fixation of severe craniofacial injuries, where cosmetic outcome is a major concern, would benefit from a fixation technology capable of holding bone fragments in alignment while they heal and then disappear to avoid the sequela associated with a persistent foreign body response (FBR). Despite incentives to create such a fixation technology, there are no adhesives in clinical use for craniofacial fracture repair that exhibit these properties.

Developing a bone adhesive is a challenging problem. The adhesive must bond to bone in the wet, bloody, ionically-rich environment of the extracellular space, and neither shrink nor swell significantly during or after curing. The delivery method must be simple. The adhesive must be non-cytotoxic as delivered as well as during subsequent breakdown. The adhesive must allow for the precise repositioning of the fractured bones, as well as maintaining their three-dimensional alignment and fixation during healing. The fixation should be robust to allow the patient free mobility after reconstruction. Inflammation should be commensurate with the healing process and subside as the adhesive is resorbed. The adhesive should not interfere with osteosynthesis at the fracture site or induce changes in the adjacent bone or soft tissues. Finally, the adhesive should eventually disappear to extinguish the FBR and its associated sequela. With regard to the currently available technology, a clinically satisfactory solution requires trade-offs in these specifications; cyanoacrylate adhesives have high fixation strengths but are not degradable and exhibit cytotoxicity, while bioadhesives, like fibrin, are degradable but lack sufficient bond strength [6].

Numerous aquatic organisms, freshwater and marine, produce underwater adhesives highly adapted to specific functions ranging from sessile fixation, to prey capture, to construction of protective shelters. In the latter category, the Sandcastle worm (*Phragmatopoma californica*) assembles fragmented bits of seashells into tubular underwater dwellings with minute dabs of a proteinaceous

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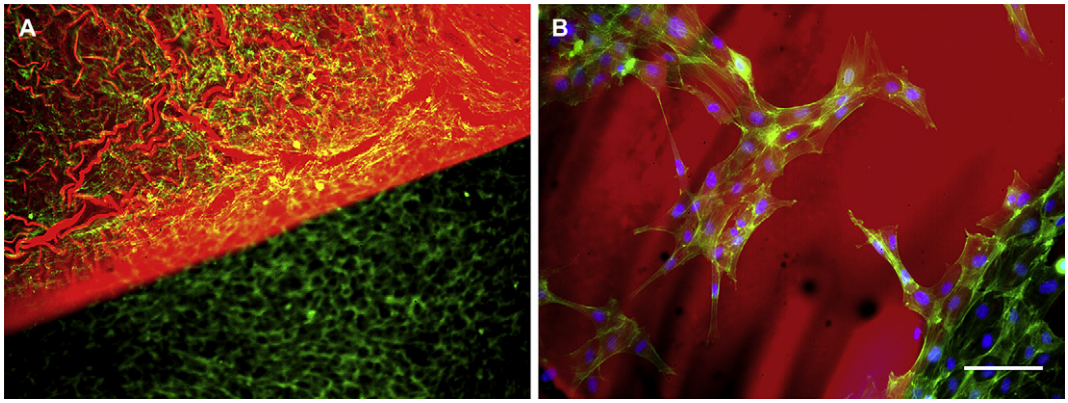

Fig. 1. *In vitro* cytocompatibility. After 48 h exposure to the adhesive complex coacervate, osteoblast viability (live/dead) and cell morphology (phalloidin-FITC staining) assays showed cell contact spreading and proliferation. A) Adhesive associated with osteoblast attachment on tissue culture polystyrene, showing homogeneous staining both on the polystyrene and the adhesive. B) Magnified view from A, showing osteoblast attachment directly to the adhesive. Adhesive (red), actin (green), and DAPI (blue). Scalebar = 100 μm.

bioadhesive [7]. The sandcastle glue is adapted precisely for joining biogenic minerals together underwater. From this perspective, it is an excellent model for a biomimetic adhesive for crainiofacial fracture repair.

The natural glue is comprised of a set of oppositely charged proteins complexed with calcium and magnesium ions. The positively charged proteins contain basic residues with amine side chains; while the negatively charged proteins contain extensive segments of acidic phosphoserine residues [8,9]. In total, approximately 20 and 30 mol% of the adhesive amino acid residues are basic and acidic, respectively. Analogs of the adhesive proteins were created with a set of oppositely charged synthetic copolyelectrolytes containing the same chemical side chains (phosphates and amines) in the same mol% as the natural proteins. When mixed in water at physiological pH and with roughly equimolar ratios of positive and negative charges the synthetic copolyelectrolytes associated and phase separated into a dense fluid state called a complex coacervate [10,11].

As the foundation for underwater adhesives, such complex coacervates have several unique and ideal properties, which have been exploited by Sandcastle worms and related organisms for a few hundred million years. They are phase separated fluids that do not disperse when delivered underwater, yet readily adhere to and spread on wet biogenic mineral surfaces. Relevant to medical adhesive needs and specifications, their rheological properties allow them to be delivered through fine gauge cannulas. They are also self-organized in water from pre-polymerized components so significant volume changes, in theory, are not expected to occur upon cure, and repaired tissues are not exposed to toxic solvents, reactants, reaction by-products, or heat generated by exothermic *in situ* polymerization during cure. The underwater bond strengths of the adhesive complex coacervates are greater than the reported bond strengths of natural bioadhesives [12,13].

Here, using a combination of *in vitro* and *in vivo* approaches, we evaluated a synthetic bioadhesive modeled after the natural underwater adhesive created by the sandcastle worm for cytocompatibility *in vitro* and biocompatibility *in vivo* using a rat calvarial model [14] over a 12 week indwelling period.

2. Methods

2.1. Bioadhesive formulation

Adhesive complex coacervates were prepared as previously reported [11]. Briefly, a commercial non-gelling collagen hydrolysate was amine-modified with ethylenediamine dihydrochloride. A 50 mg/ml aqueous solution of the aminated gelatin at pH 7.4 was added while stirring to a 50 mg/ml aqueous solution of polyphosphodopa containing Ca^{2+} (Ca^{2+}/phosphate sidechain = 0.2) at pH 7.4. The mixture was stirred for 30 min and the coacervate phase was collected by centrifugation. Sodium periodate was added to the gelatin complex coacervate at 1:2 ratio to dopa side chains to initiate crosslinking of the adhesive complex coacervate.

2.2. Cell culture

Direct and indirect contact cell culture assays were used to evaluate cytocompatibility of the adhesive complex coacervates using a mouse osteoblast (MC3T3-E1) cell line (ATCC, Manassas VA). Briefly, 1 μl of the adhesive was applied into empty wells of 12-well tissue culture plate, and flattened using a Teflon sheet (width 6 mm). 1 ml of α-minimum essential medium (α-MEM) with oxidant $NaIO_4$ at molar ratio of 1:2 to Dopa side chains in the adhesive was added to initiate oxidization. Osteoblasts were plated on top of the bioadhesive at a density of 200,000 cells/well in the α-MEM containing 10% fetal bovine serum, 50 μg/ml penicillin and 50 μg/ml ascorbic acid at 37 °C for 48 h. The osteoblasts were then stained to determine cell viability by live/dead staining (Invitrogen L3224, Carlsbad CA) and imaged with a Nikon Eclipse TE300 inverted scope at 10×. Following imaging, the cells were fixed by immersion in 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4 for one hour and then stored in PBS with 0.01% NaN_3. Cell morphology was assessed with immunoflourescent histochemistry using FITC labeled phalloidin (Invitrogen F432, Carlsbad CA), to image actin microfilaments and DAPI to label cell nuclei. Cytocompatibility was evaluated by the presence of viable cells with spread morphology in contact with the surface of the bioadhesive. Cell cultures without the addition of bioadhesive served as controls.

2.3. Surgical procedures

All animal procedures were conducted in accordance with the University of Utah Institutional Animal Care and Use Committee (IACUC). Adult male Sprague Dawley rats (200–225 g) were anesthetized via an intraperitoneal injection of ketamine (65 mg/kg), xylazine (7.5 mg/kg), and acepromazine (0.5 mg/kg). After reaching a full level of anesthesia as assessed using tail pinch, animals' heads were shaved, and disinfected with isopropanol and betadyne, followed by transfer to a stereotaxic frame. A midline incision extending the length of the skull was made, and the skin was retracted and the fascia was reflected laterally with a scalpel blade. The surface of the skull bone was dried with cotton-tipped applicators.

To evaluate biocompatibility of the adhesive complex coacervates and its fixation to normal bone in the absence of bone injury, 1 μl of adhesive was placed directly on the surface of the skull ($n = 12$) and allowed to oxidize. The scalp incision was then closed with 5/0 silk sutures, and the animals were allowed to recover.

To evaluate the biocompatibility in a fracture model, another group of animals had a 3 mm diameter burr hole positioned 3.2 mm posterior to bregma, and 2 mm lateral to bregma cut using a pneumatically-driven trephine under stereotactic control. The circular piece of bone from the craniotomy was retrieved, irrigated with sterile PBS, replaced in the circular hole and aligned in place with the aid of a stereomicroscope. Approximately 1 μl of adhesive was pipetted into the circular gap ($n = 8$), while others that served as controls had no adhesive to fix and maintain alignment of the circular piece of bone ($n = 4$; see Fig. 4). In both cases, the scalp incision was closed with 5/0 silk sutures, and the animals were allowed to recover and freely move about until sacrifice.

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