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Rapidly polymerizing injectable click hydrogel therapy to delay bone growth in a murine re-synostosis model

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ABSTRACT

Craniosynostosis is the premature fusion of cranial sutures, which can result in progressive cranial deformations, increased intracranial pressure, and restricted brain growth. Most cases of craniosynostosis require surgical reconstruction of the cranial vault with the goal of increasing the intracranial volume and correcting the craniofacial deformities. However, patients often experience rapid post-operative bone regrowth, known as re-synostosis, which necessitates additional surgical intervention. Bone morphogenetic protein (BMP) inhibitors have tremendous potential to treat re-synostosis, but the realization of a clinically viable inhibitor-based therapeutic requires the development of a delivery vehicle that can localize the release to the site of administration. Here, we present an in situ rapidly crosslinking injectable hydrogel that has the properties necessary to encapsulate co-administered proteins and demonstrate that the delivery of rmGremlin1 via our hydrogel system delays bone regrowth in a weanling mouse model of re-synostosis. Our hydrogel is composed of two mutually reactive poly(-ethylene glycol) macromolecules, which when mixed crosslink via a bio-orthogonal Cu free click reaction. Hydrogels containing Gremlin caused a dose dependent inhibition of bone regrowth. In addition to craniofacial applications, our injectable click hydrogel has the potential to provide customizable protein, small molecule, and cell delivery to any site accessible via needle or catheter.

1. Introduction

Craniosynostosis is the pathologic premature fusion of the cranial sutures early in development, affecting nearly 1 in 2000 children [1–4]. If left untreated, craniosynostosis can result in progressive craniofacial deformities, restricted brain growth, and an increase in intracranial pressure (ICP), which may cause central nervous system complications such as blindness, deafness, seizures, and in extreme cases death [5–9]. The standard treatment for most cases of craniosynostosis is complex cranial vault reconstruction, wherein surgeons remove the majority of the calvaria and reshape the bones to increase the intracranial volume and correct the craniofacial deformities. In up to 10–40% of children, bone rapidly re-grows following surgery, resulting in a condition called re-synostosis [10,11]. This re-synostosis can result in a subsequent increase in intracranial pressure and other nervous system complications. These patients frequently undergo subsequent cranial vault reconstructions, which are associated with a high incidence of life threatening complications including meningitis, encephalitis, and intracranial hemorrhage [12,13]. Despite the frequency and severity associated with the treatment of re-synostosis, there are no clinically viable therapies to control or prevent rapid post-operative cranial fusion.

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The earliest treatment for re-synostosis involved placing a variety of physical barriers in the spaces created during surgery. These procedures were abandoned because these materials resulted in an increase in infections with no substantial delay in bone growth [14]. As a result, any therapy designed to delay re-synostosis must target the biological processes that are responsible for rapid bone formation. Prior work with both rabbit and adult murine models has attempted to prevent post-operative bone growth [15–18]. These methods have primarily used collagen-based vehicles or ex-vivo based therapies to control the post-operative bone growth.

One of the major challenges when delivering any biologically active molecule is rapid diffusion away from the sight of administration. This makes it impossible to sustain therapeutic concentrations of the active molecules via bolus injection within the defects created during cranial vault surgery. Given their ability to conform to the shape of tissue defects and localize the continuous release of proteins to the site of administration, injectable in situ crosslinking hydrogels are ideal platforms for the sustained delivery of biologically active molecules. Injectable hydrogels are composed of mutually reactive soluble precursors that react in situ to form insoluble networks. In addition, due to their high water content, hydrogels are well suited for the delivery of proteins and other biomolecules that can be denatured upon contact with hydrophobic surfaces [19].

Traditional gelation chemistries that are based on free radical polymerization, Michael addition, or amide bond formation, are either too slow to encapsulate co-delivered proteins before they diffuse away from the site of administration, or use initiators and precursors that react with cellular components and can be toxic to neuronal and skeletal tissues [20–26]. “Click chemistry” and other bio-orthogonal gelation mechanisms have great promise for in situ hydrogel formation, due to their rapid polymerization kinetics and low reactivity with cellular components. However, the development of catalyst-free click chemistry-based gelation mechanisms for protein release in vivo remains a major challenge. Thus, there is great interest in developing new injectable hydrogels based on click chemistry that can deliver proteins in vivo [27,28].

Bone morphogenetic protein (BMP) antagonists are attractive therapeutic proteins for controlling the rate of bone growth as they are normally secreted extracellularly to control the activity of BMP [29,30]. BMP is produced by osteoblast-lineage cells and acts in an autocrine/paracrine manner to stimulate bone formation. Activity of BMP is tightly controlled in vivo via a complex set of regulatory strategies involving availability of inhibitors, receptor subunits, and intracellular signaling mediators. Endogenous BMP inhibitors are secreted extracellularly and function by binding to the target BMP molecule and in turn preventing BMP receptor activation. BMP inhibitors are differentially expressed during the rapid bone growth that occurs following the surgical removal of the posterior frontal suture in weanling mice [31]. One of these inhibitors, Gremlin is an attractive BMP inhibitor for use in delaying re-synostosis as it is up regulated prior to the BMP2 and BMP4 mediated mineralization seen in a weanling murine model of re-synostosis [31]. In contrast, the widely studied antagonist Noggin was upregulated following the completion of mineralization of the cranial defect [15,16,31,32]. As a result, we hypothesized that Gremlin would be a more effective inhibitor as it was involved in the cartilage to mineralized tissue transition early in defect healing.

Here, we present a bio-orthogonal injectable hydrogel that is designed to crosslink to completion in less than 2 min and should thus have the gelation kinetics needed for in situ encapsulation and subsequent delivery of the BMP inhibitor rmGremlin1. In order to achieve such rapid network formation, we synthesized multivalent poly(ethylene glycol) precursors (Fig. 1a, 1 & 2) that form an insoluble network upon mixing (Fig. 1a, 3) via the ring-strain promoted Cu-free click reaction between dibenzylcyclooctynes (DBCO) and azides. Our hydrogel system also contains ester linkages to ensure that the system is ultimately degraded and excreted, which we anticipate will happen on the timescale of weeks. We selected the DBCO-azide reaction for in situ gelation because it is two orders of magnitude faster than previously reported click-based gelation mechanisms, proceeds under physiological conditions, and is non-toxic to cells [33]. Using a cranial defect over the posterior frontal suture of weanling mice, we investigated the ability of our hydrogel system to release therapeutic concentrations of active Gremlin1 by examining the effects of sustained release of the protein over 14 days on inhibition of the rapid post-operative bone growth that occurs with this model.

2. Materials and methods

2.1. Synthesis of tetraethylene glycol methacrylate (TEGMA)

Tetraethylene glycol (5.0 g, 25.7 mmol), and pyridine (2.0 g, 25.3 mmol) were added to anhydrous dichloromethane (DCM) (100 mL) in a 250 mL flask and stirred for 30 min at 0 °C. Methacryloyl chloride (2.6 g, 25 mmol) was added drop-wise to the stirred solution. The reaction was allowed to stir at 0 °C for 2 h, and then at room temperature (rt) for an additional 2 h. The reaction was then concentrated via rotary evaporation, re-suspended in ethyl acetate, and finally evaporated onto silica gel. The mono methacrylate product was separated from the di-methacrylate byproduct and starting material via flash silica gel chromatography on silica gel using a mixture of ethyl acetate and hexanes (7:3). 1H NMR (300 MHz, $CDCl_3$) δ 2.01 (t, $J = 6.0$ Hz, 3H,CH_3), 2.82(t, $J = 6.0$ Hz, 1H,OH), 3.49–3.65 (m, 16H, 8 × CH_2), 6.48 (m, 2H, CH_2=C); 13C NMR (75.5 MHz, $CDCl_3$) δ 167.32 (C=O), δ 136.0 (C=CH_2), 125.87 (CH_2=C), 61.51 (CH_2OH), 69.91 (CH_2), 70.21 (CH_2), 70.46 (CH_2), 70.52 (CH_2), 70.56 (CH_2), 72.43 (CH_2), 39.23 (C), 17.83 (CH_3).

2.2. Synthesis of tetraethylene glycol mono 4-methylbenzenesulfonate

Tetraethylene glycol (5.0 g, 25.7 mmol) and pyridine (2.0 g, 25.3 mmol) were added to anhydrous dichloromethane (100 mL) in a 250 mL flask and stirred for 30 min at 0 °C. A solution of 4-toluenesulfonylchloride (4.75, 20 mmol) in 30 mL DCM was added drop-wise via syringe pump to the flask. The reaction mixture was then stirred for 2 h at 0 °C, then another 4 h at rt. The reaction mixture was then poured into ice water and the organic layer was separated then washed 2× with brine and dried over $MgSO_4$ before being concentrated via rotary evaporation. The crude product was then re-suspended in ethyl acetate and evaporated onto silica gel. The mono tosylated product was separated from the di-tosylated byproduct and starting material via flash silica gel chromatography using a mixture of ethyl acetate and hexanes (6:4). 1H NMR (300 MHz, $CDCl_3$) δ 2.33 (s, 3H, CH_3), 2.89 (t, $J = 6.0$ Hz, 1H, OH), 3.42–3.70 (m, 14H, 7 × CH_2), 4.00–4.12 (m, 2H, CH_2OTs), 7.24 (d, $J = 8.0$ Hz,2H, 2 × Hm), 7.68 (d, $J = 8.0$ Hz, 2H, 2 × Ho); 13C NMR (75.5 MHz, $CDCl_3$) δ 21.41 (CH_3), 61.40 (CH_2OH), 68.44 (CH_2OTs), 69.17 (CH_2), 70.10 (CH_2), 70.22 (CH_2), 70.41 (CH_2), 70.46 (CH_2),72.34 (CH_2), 127.73 (2 × CHo), 129.68 (2 × CHm), 132.76 (C), 144.68 (C).

2.3. Synthesis of tetraethylene glycol mono azide

Sodium azide (2.0 g, 30.76 mmol) was added to a solution of tetraethylene glycol mono 4-methylbenzenesulfonate (2.0 g, 5.74 mmol) in dimethylformamide (100 mL) at room temperature. The reaction mixture was stirred overnight at 90 °C. The reaction was then filtered and concentrated via rotary evaporation. The crude product was added to cold water and extracted with ethyl acetate (4 × 100 mL). The combined organic layers were then dried over $MgSO_4$ and concentrated under vacuum. The viscous liquid was then purified by flash column chromatography on silica gel using a mixture of ethyl acetate and hexanes (6:4) to yield the desired product as a colorless oil (1.63 g, 81%): 1H NMR (300 MHz, $CDCl_3$) δ 2.91 (t, $J = 6.0$ Hz, 1H,OH), 3.30 (t, $J = 5.0$ Hz, 2H, CH_2N_3), 3.49–3.65 (m, 14H, 7 × CH_2); 13C NMR (75.5 MHz, $CDCl_3$) δ 50.54 (CH_2N_3), 61.51 (CH_2OH), 69.91 (CH_2), 70.21 (CH_2), 70.46 (CH_2), 70.52 (CH_2), 70.56 (CH_2), 72.43 (CH_2).

2.4. Synthesis of azido tetraethylene glycol methacrylate (ATEGMA)

Tetraethylene glycol mono azide (2.0 g, 9.13 mmol) and pyridine (2.0 g, 25.3 mmol) were added to anhydrous dichloromethane (DCM) (100 mL) in a 250 mL flask and stirred for 30 min at 0 °C. Methacryloyl chloride (2.6 g, 25 mmol) was added drop-wise to the stirred solution. The reaction was allowed to stir at 0 °C for 2 h, and then at room temperature for an additional 2 h. The reaction was then concentrated via rotary evaporation, re-suspended in ethyl acetate, and finally evaporated onto silica gel. The mono methacrylate product was separated from the starting material via flash silica gel chromatography on silica gel using a mixture of ethyl acetate and hexanes (4:6 v/v). 1H NMR (300 MHz, $CDCl_3$) δ 2.01 (t, $J = 6.0$ Hz, 3H,CH_3), 2.91 (t, $J = 6.0$ Hz, 1H,OH), 3.30 (t, $J = 5.0$ Hz, 2H, CH_2N_3), 3.49–3.65 (m, 14H, 7 × CH_2), 6.48 (m, 2H, CH_2=C); 13C NMR (75.5 MHz, $CDCl_3$) δ 167.32 (C=O),136.0