Implantable biomaterial based on click chemistry for targeting small molecules

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A B S T R A C T
Specific and targeted delivery of medical therapies continues to be a challenge for the optimal treatment of multiple medical conditions. Technological advances permit physicians to target most sites of the body. However, after the intervention, physicians rely on systemic medications that need frequent dosing and may have noxious side effects. A novel system combining the temporal flexibility of systemic drug delivery and the spatial control of injectable biomaterials would improve the spatiotemporal control of medical therapies. Here we present an implantable biomaterial that harnesses in vivo click chemistry to enhance the delivery of suitable small molecules by an order of magnitude. The results demonstrate a simple and modular method to modify a biomaterial with small molecules in vitro and present an example of a polysaccharide modified hours after in vivo implantation. This approach provides the ability to precisely control the moment when biochemical and/or physical signals may appear in an implanted biomaterial. This is the first step towards the construction of a biomaterial that enhances the spatial location of systemic small molecules via in vivo chemical delivery.

1. Introduction

The specific and targeted delivery of therapies in post-operative pain management [1], localized antibiotics [2,3] or chemotherapeutic delivery continues to be a challenge [4,5]. In addition, modern practitioners face the increasing incidence of antibiotic-resistant pathogens [6–9], an aging patient population with multiple co-morbidities [10,11], polypharmacy [12,13] and higher use of prescription narcotics [14]. All of this means that there is a critical need for solutions that lead to medically efficient, cost-effective strategies for local therapeutic delivery with minimal side effects.

Modern drug delivery systems attempt to optimize the local and timely (spatial and temporal) delivery of therapeutics [1,3,4,15]. Existing biomaterials can serve as depots of medication through diffusion, affinity, immobilization or degradation [1]. However, most biomaterials cannot be modulated or modified after implantation, and usually exhibit an initial burst of activity shortly after implantation [1,16]. These issues limit the application of biomaterials for multiple medical conditions that require the doses to be tailored at different time points [16,17] or for which the most effective therapeutic agent is identified hours or days after implantation, e.g. after culture or pathology results are obtained.

Historically, cycloaddition reactions have been a powerful tool in the chemical synthesis of complex natural products [18]. One cycloaddition reaction, the inverse-electron-demand Diels–Alder reaction, has been used for several in vivo click chemistry applications [19–21] and one of the most studied applications is for pretargeted tumor imaging [22–24]. Briefly, this technique involves the chemical conjugation of a monoclonal antibody (mAb) specific to a tumor marker with a trans-cyclooctene (TCO) moiety. The mAb–TCO conjugate is injected intravenously into a mouse growing a subcutaneous tumor. After an elapsed period of time, a chemical probe containing a tetrazine (Tz) moiety with a handle for radiolabeling, for example the SPECT emitter, indium-111 chelated by 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (111In-Tz; Fig. 1b, 2) is injected intravenously to the animal with subsequent localization of the radioactivity at the tumor. This demonstrates that TCO and Tz reagents are able to react with each other in vivo at the surface of a tumor [22]. However, the need for mAbs and multi-day interventions hinder the application of this promising technology to other areas of medical research.
We envision that a new system that combines the temporal flexibility of systemic drug delivery and the spatial control of injectable biomaterials could enhance the localization of small molecules (Fig. 1a). Here we report the design and construction of an alginate polymer (TCO-gel; Fig. 1b, 1) that was covalently modified to incorporate TCO molecules to its sugar backbone (Fig. 1b). Furthermore, we report the in vitro and in vivo interactions of this material with $^{111}$In-Tz molecules (Fig. 1b) [22]. The hypothesis is that TCO-gel will react with circulating $^{111}$In-Tz molecules through an inverse electron demand Diels–Alder reaction in a bioorthogonal fashion localizing the Tz molecules and their radioactive cargo to the TCO-gel.

2. Materials and methods

2.1. Materials

All reagents and nuclear magnetic resonance (NMR) solvents were purchased from Sigma–Aldrich (St. Louis, MO), unless otherwise noted. Chemical synthesis of (R,E)-N-(2-aminoethyl)-2-(cyclooct-4-en-1-yloxy)acetamide, the precursor of TCO-gel, was contracted to MBMR Bioslabs Inc. (Brooklyn, NY). Silica gel was purchased from Silicycle (Quebec, Canada), while preparative thin-layer chromatography (TLC) plates (20 × 20 cm; 1000 μm in thickness) were purchased from Analtech (Newark, DE). Ultrapure (UP) MVG sodium alginate, a medium-viscosity (>200 mPa s) sodium alginate where a minimum of 60% of the monomer units are guluronate, was purchased from ProNova BioPharma ASA (Lysaker, Norway). $^{[111]}$Indium chloride solution was purchased from PerkinElmer (Waltham, US). Dulbecco’s phosphate buffered saline (DPBS, without calcium and magnesium) was obtained from Cellgro (Mediatech, Manassas, VA, USA) and had the following formulation (g l$^{-1}$): KCl, 0.20; KH$_2$PO$_4$, 0.20; NaCl, 8.00; Na$_2$HPO$_4$ (anhydrous), 1.15.

2.2. Syntheses of click chemistry components

A detailed description of the synthesis of [(R,E)-N-(2-aminoethyl)-2-(cyclooct-4-en-1-yloxy)acetamide], the precursor of TCO-gel, is provided in the Supplementary Information. Tz was prepared according to published procedures [22]. The $^1$H-NMR spectrum matched the one previously published and high-resolution mass spectrometry (HRMS) data were consistent with the expected values. HRMS: m/z [M+H]$^+$ calculated for C$_{27}$H$_{29}$N$_{13}$O$_{20}$ 1278.6576, found 1278.6580.

2.3. Radiolabeling of Tz

Tz was dissolved to a concentration of 1.0 mM in 0.2 M ammonium acetate (pH 7.0) and stored at −80 °C before use. An aliquot of Tz was combined with a suitable amount of $^{[111]}$indium chloride solution (approximately 700 kBq of $^{[111]}$In per nmol of Tz) and incubated for 10 min at 37 °C under gentle agitation. Next, 5 μl of 10 mM diethylenetriaminepentaacetate was added and the solution was incubated for an additional 5 min. Typically, a quantitative labeling yield and a radiochemical purity greater than 97% were obtained; confirmed by radio-TLC and radio-high-performance liquid chromatography. For animal experiments, the solution of $^{[111]}$In-Tz was diluted with sterile saline.

2.4. Alginate chemical modification

Each gram of UP MVG alginate was combined with 176 μmol of TCO-amine under standard carbodiimide chemistry conditions, as previously described for arginine–glycine–aspartic acid (RGD) [25] and glycine–histidine–lysine (GHK) [26] incorporation. The alginate product was then purified by dialysis against deionized water containing decreasing salt concentrations for 4 days, frozen and lyophilized for 5–10 days until dry. A 2.5% alginate solution was obtained by adding DPBS, and alginate gels were fabricated by the addition of calcium. Covalent modification of alginate was confirmed through $^1$H-NMR studies (refer to Supplementary Information for spectra). The same protocol without the TCO addition was used for the construction of control gels. The in vitro and in vivo studies were done with TCO-gel from the exact same batch and used on the same day to minimize any variations in loading amount or loading efficiency.

For in vitro experiments, 800 μl of 2.5% alginate solution was mixed with 200 μl of supersaturated Ca(SO$_4$)$_2$ solution (0.21 g of Ca(SO$_4$)$_2$ per ml of double-distilled water (ddH$_2$O)). The solutions were mixed for 30 s using a three-way stopcock to achieve a final

Fig. 1. Experimental description. (a) The method consists of the initial injection of a solid carrier covalently attached to TCO molecules. This is followed by a systemic injection of small molecules synthetically coupled to tetrazine moieties. When the two entities are in close proximity, they react irreversibly attaching the therapy to the solid carrier and thus localizing the small molecule. (b) Two molecular probes were utilized for the study: TCO-gel, 1 and $^{111}$In-Tz, 2.
alginate concentration of 2%. The mixture was allowed to gel between two glass plates in a custom-made plastic model and incubated for 20 min at room temperature. The entire volume had a uniform appearance consistent with gelation. The discs were picked up with a spatula and weighed individually. Typically, a preemade disc weighed approximately 100 mg and had roughly the following dimensions: 8 mm (diameter) and 2 mm (height).

For in vivo use, the 2.5% alginate gel solution and the super-saturated calcium sulfate solution were mixed rapidly in the same proportions as mentioned above and immediately injected to the animal in the desired amount.

2.5. In vitro quantification of TCO-gel

The reactivity of control gel and TCO-gel discs were tested in ddH2O. Using 2.0% (w/v) alginate solution in DPBS and calcium ions as crosslinkers, TCO-gel and control gel discs were created [25]. A premade disc (mean weight 0.10 g (0.06–0.12 g)) was placed in a test tube and a solution containing 111In-Tz (1.04 MBq, 0.47 nmol in 0.20 ml of ddH2O) was added. Radioactivity levels were confirmed using a Capintec Dose Calibrator (Capintec, Inc.). The discs were then allowed to sit at room temperature for either 10 min (n = 1) or 14 h (n = 3). They were washed three times with 0.25 ml of ddH2O and the radioactivity was measured again.

2.6. Animals and animal handling

Female BALB/c mice (20–25 g body weight, Charles River Laboratories) were used for these studies. All animals were handled in accordance with a protocol approved by the University of California, Davis, Institutional Animal Care and Use Committee and experiments were performed according to the Principles of Laboratory Animal Care (NIH publication 85–23, revised 1985).

2.7. In vivo biodistribution study

Either control gel or TCO-gel was injected subcutaneously at each flank area of the subject (mean weight 0.22 g (0.10–0.29 g)). Approximately 3–4 h after gel injection, 111In-Tz dissolved in 0.9% NaCl (50 µl, mean dose 1.63 MBq (1.45–1.79 MBq), mean 0.88 nmol (0.78–1.01 nmol)) was injected intravenously (i.v.) via a catheter into the tail vein of the mouse. At selected time points (1, 4, 24, 48 h), mice (n = 3 per time point) were euthanized and dissected, and tissues of interest, such as blood, gallbladder, liver, heart, kidneys, pancreas, spleen, lungs, stomach, small intestine, large intestine, bladder, skin, muscle, bone, and brain, were harvested, together with the gels and surrounding tissues (control gel, TCO-gel). Solid tissues were washed with deionized water to remove excess blood and all collected material was weighed. The radioactivity associated with each sample was measured using a Wallac 1470 Wizard gamma counter (PerkinElmer, Inc.). Radioactivity uptake (decay corrected) is presented as percent injected dose per gram (%ID g−1). The results are expressed as mean ± SD.

2.8. SPECT/CT imaging study

Either control gel or TCO-gel was injected subcutaneously at each flank area of the subject (0.23 g for control gel and 0.17 g for TCO-gel). Approximately 3 h after injection of the gel, 111In-Tz dissolved in 0.9% NaCl (90 µl, 38.8 MBq, 21 nmol) was injected i.v. via a catheter into the tail vein of the mouse. At 4, 24 and 48 h after 111In-Tz injection, the mouse was anesthetized with 1–2% isoflurane and positioned in the prone position on an animal bed equipped with a nose cone for anesthesia and tubing with warm water to maintain adequate body temperature. SPECT imaging was performed with a SPECT/CT Inveon Preclinical Imaging Station (Siemens Healthcare) for 60 min using a 5-pin hole collimator. Immediately after SPECT acquisition, CT images were acquired. Coregistration of SPECT and CT images was performed using fiducial markers and the Inveon Research Workplace (IRW) software (Siemens Healthcare).

2.9. Statistics

Group variation is described as the mean ± one standard deviation. For in vitro and biodistribution studies, single groups were compared with a two-tailed paired t-test. Groups with p < 0.05 were considered significantly different. Microsoft Excel version 12.8.9 was used for graphs and statistical calculations.

3. Results

3.1. Fabrication and in vitro characterization of click chemistry-modified biomaterial

The TCO-gel discs maintained significantly more activity than the control gels (Fig. 2). The level of activity of the control gel was equivalent to 0.86 ± 0.21 nmol g−1 of 111In-Tz (n = 3, 14 h), while the amount of radioactive molecules bound covalently to TCO-gel was 2.94 ± 0.05 nmol g−1 after 14 h of incubation (n = 3). Comparison between the control and the TCO-gel revealed a statistical significant difference (p-value < 0.05) as measured by paired t-test. These results confirmed that this concept was viable in a test tube.

In addition, a single sample was stopped after just 10 min. The level of activity of the control gel was equivalent to 0.75 nmol g−1 of 111In-Tz (n = 1). On the other hand, the amount of radioactivity at TCO-gel at 10 min was 1.03 nmol g−1 (n = 1).

3.2. In vivo characterization of local delivery

To assess the in vivo relevance of the observed in vitro binding, BALB/c mice were injected at the flank area with subcutaneous injections of either control gel or TCO-gel. After approximately 3 h, the mice received a tail-vein i.v. injection of 111In-Tz. The levels of radioactivity delivered to each organ or hydrogel site were determined by biodistribution studies at 1, 4, 24 and 48 h with 3 mice per time point (Fig. 3). The mean weight of gel retrieved was 0.15 ± 0.04 g (69% of injected gel). In order to obtain an estimate of the remaining 30% of gel injected, we carefully collected the tissue surrounding each gel (0.12 ± 0.04 g). In addition, SPECT images of one mouse were taken at 4, 24 and 48 h to visualize the spatial distribution of indium-111 (Fig. 4).

Our results indicate that we achieved an in vivo click reaction with delivery of greater than 4% intravenous ID g−1 in the subcutaneous space of a murine model at 1 h. In contrast, the radioactivity level delivered to unmodified muscle or skin tissues was <0.3% ID g−1. The level of radioactivity at the control gel and surrounding tissue was <1.8% ID g−1 at 1 h and declined rapidly to background levels thereafter (<0.4% ID g−1). In contrast, the amount of radioactivity at the TCO-gel site was maintained to a level above 1% ID g−1 even after 48 h. The difference between the groups was statistically significant at all time points (Figs. 3a and 4).

Our results are also consistent with previous literature reports, which indicate that 111In-Tz is excreted renally and has a short in vivo half-life of about 10 min [22]. The major activity, other than TCO-gel, was found at the kidneys (2.3% ID g−1) and the bladder (1.1% ID g−1) at 1 h. The level of activity circulating in the blood at 1 h was comparable to the level in the control gel (1.7% ID g−1). The activity at the lungs was 0.7% ID g−1 at 1 h and declined rapidly thereafter. All other organs examined were <0.5% ID g−1 at 1 h and...
then declined to background levels. Also, $^{111}$In-Tz appeared unable to cross the blood–brain barrier, with the brain having an activity level of <0.1% ID g$^{-1}$ at 1 h.

4. Discussion

Since its emergence in 2001, click chemistry [19,27,28] has been applied to many fields of modern chemical science. One of its most recent incarnations is for in vivo pretargeted nuclear imaging approaches. The technique entails the delivery of a molecular load through antibodies to a subcutaneous tumor [20,22,23]. Tumors, which are well known to have a leaky vasculature and increased blood flow [1,29], likely maximize the exposure of the mAb–TCO to the Tz radioprobe. Moreover, while a mAb can provide exquisite localization, the system has some limitations. Antibodies require: (i) an identifiable and accessible target; (ii) specificity to said target; (iii) the ability to be chemically modified to carry a TCO cargo and maintain specificity [5,22]; and (iv) rapid clearance from the blood stream of unbound mAbs to minimize unwanted side effects [24]. The platform we describe in this report circumvents the need for mAbs, providing a modular and flexible platform for in vivo small molecule delivery.

Previous methods to assess the incorporation of small molecules to an alginate polymer use radiolabeled molecules as tracers and a comparison of the activity between the product and the initial reaction solution [25]. However, the trans-chemical bond of TCO is known to isomerize to the less reactive cis-bond under a number of conditions [30]. Since the purification of the alginate polymer after the amidation reaction involves multi-day dialysis...
and a lyophilization step, it is unlikely that the initial incorporation of TCO to the alginate is representative of the number of TCO molecules that resisted isomerization during the reaction conditions needed to obtain TCO-gel. Thus we developed an in vitro protocol to quantify the functional loading of TCO molecules in TCO-gel.

Careful evaluation of the in vitro results reveals that a small amount of $^{111}$In-Tz binds the control gel and does not seem to increase over time. The most likely explanation is that non-specific forces between $^{111}$In-Tz and the polysaccharide backbone of alginate lead to this finding. The affinity of cationic small molecules to alginate has been well characterized in the literature [1]. In contrast, the amount of radioactivity bound to TCO-gel seems to increase over time, and after 14 h there is a statistical significant difference in the amount of $^{111}$In-Tz bound between TCO-gel and control gel. This encouraging piece of data led us to test our platform in an animal model.

Our results show that $^{111}$In-Tz, a molecule with expedient renal clearance and an in vivo half-life of 10 min, can be captured by our platform and its local concentration can be increased by an order of magnitude (0.3–4.0% ID g$^{-1}$). Consistent with our in vitro studies, there is a small level of radioactivity present at the control gel, which seems to correlate with the blood radioactivity level and decreases rapidly, as one would expect for non-specific binding forces. The level of radioactivity at the experimental site of TCO-gel at 1 h is significantly higher and is maintained above background for 2 days. The results of the imaging and biodistribution studies support the enhanced localization of systemic $^{111}$In-Tz to TCO-gel.

Our results have elucidated the following: (i) alginate is a suitable polymer backbone to carry out vivo chemistry; (ii) the perfusion of a biomaterial in healthy subcutaneous space allows the local delivery of a compound ($^{111}$In-Tz) even with an in vivo half-life of 10 min [22]; and (iii) TCO-gel can be implanted for hours and be an adequate partner for in vivo chemistry. Nonetheless, the current proof of concept also contains several limitations. The small molecules delivered remain covalently attached to the macromolecular delivery device (TCO-gel).

While the vast majority of therapeutic agents work in solution, there are several reports of the retained in vivo activity of small molecules and proteins after covalent linkage to biomaterials. For instance, it has been shown the covalent modification with vancomycin of titanium alloys [31–35] and bone grafts [36–39] inhibit bacterial biofilm formation in vitro and in vivo [2,40]. Others have reported the benefits of antimicrobial peptides that are covalently immobilized onto biomaterial surfaces [41]. Furthermore, multiple studies have shown that signaling proteins such as vascular endothelial growth factor [42], granulocyte macrophage-colony stimulating factor [43] and interferon-gamma [44] can be successfully immobilized and maintain their activity. These studies illustrate the benefits of biomaterials that are covalently modified with therapeutic agents. The common denominator of these strategies is that the therapeutic agents have extracellular targets. Further studies are needed to expand the use of the platform presented to therapeutic agents with intracellular targets and are currently underway.

In addition, further studies are required to determine the in vivo degradation pattern of TCO-gel. Alginate hydrogel is a biocompatible and cytocompatible material that has found wide use in biomedical applications, including the delivery of inductive molecules [25,26], the transplantation of cells into defect sites [1] and even as devices for myocardial regeneration after heart attacks in humans [45,46]. Furthermore, alginate hydrogel can be modified to undergo breakdown or resist degradation [1], thus tailoring how long it stays in the body [47]. As these are the initial studies on TCO-gel, there are no previous literature reports for the degradation or activity of this specific modified biomaterial.

In the course of our animal studies, no untoward effects were noted in the mice that had the gel implanted for 1 week (unpublished studies). Further studies are needed to determine whether small molecules such as Tz or TCO are released from the hydrogel (e.g. by hydrolysis of the amide bond) and whether this causes any negative reactions.

Other avenues for exploration of this platform include the study of the reaction rate of TCO-gel, as well as the nature of the increase in binding with higher amounts of reactive molecules. Moreover, published work indicates that chemical optimizations can allow TCO molecules attached to antibodies to remain active in vivo for up to 3 days [30], but further studies are needed to determine how long after implantation TCO-gel is a viable in vivo chemistry reaction partner.

5. Conclusions

This investigation supports our hypothesis that an implantable scaffold can be chemically modulated through principles of click
chemistry and increase the concentration of systemic small molecule by an order of magnitude in the subcutaneous space. This is a novel concept for the control of local delivery of a molecular payload through a macromolecular device and presents proof for the spatiotemporal chemical modification of biomaterials after implantation by targeted delivery of systemic small molecules.

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J.M.M.O. contributed with conception, experimental design, grant writing and manuscript writing, and was physically involved in all experiments. M.G and M.L contributed equally with mentorship through monthly meetings. J.K.L. provided physical and intellectual support and advice on hydrogel studies. J.L.S. provided physical and intellectual support and advice during preliminary radiochemistry and preliminary animal studies. Contributing author names are in alphabetical order.

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figures 1–4, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2014.08.019.

Appendix B. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.actbio.2014.08.019.

References


