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In vitro sustained release of bioactive anti-NogoA, a molecule in clinical development for treatment of spinal cord injury

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ABSTRACT

Anti-NogoA is a promising anti-inhibitory molecule that has been shown to enhance functional recovery after spinal cord injury when delivered in rat and primate models over the span of weeks. To achieve this sustained release, anti-NogoA is typically delivered by osmotic minipumps; however, external minipumps are susceptible to infection. To address this issue, we developed a drug delivery system that consists of anti-NogoA-loaded poly(lactic-*co*-glycolic acid) nanoparticles dispersed in a hydrogel of hyaluronan and methylcellulose (composite HAMC). To optimize in vitro release, we screened formulations for improved anti-NogoA bioactivity and sustained release based on combinations of co-encapsulated trehalose, hyaluronan, $MgCO_3$, and $CaCO_3$. Co-encapsulated $MgCO_3$ and $CaCO_3$ slowed the rate of anti-NogoA release and did not influence anti-NogoA bioactivity. Co-encapsulated trehalose significantly improved anti-NogoA bioactivity at early release time points by stabilizing the protein during lyophilization. Co-encapsulated trehalose with hyaluronan improved bioactivity up to 28 d and dramatically increased the rate and duration of sustained delivery. The sustained release of bioactive anti-NogoA from composite HAMC is a compelling formulation for in vivo evaluation in a model of spinal cord injury.

1. Introduction

Spinal cord injury (SCI) affects 130,000 people each year worldwide (Thuret et al., 2006) and often results in permanent sensory and motor deficits. One promising option is treatment with anti-NogoA, an antagonist of the myelin-associated inhibitor NogoA known to cause growth cone collapse and reduce neurite outgrowth (Lee et al., 2003). Anti-NogoA has been shown to improve functional recovery when delivered by an intrathecal catheter over 14 d (Liebscher et al., 2005) and 28 d (Wu et al., 2010) in rat models, 28 d (Freund et al., 2006) in a primate model, and is currently in a phase I clinical trial (Clinicaltrials.gov, 2011; Hawryluk et al., 2008). Local delivery is preferred because anti-NogoA is an IgG (210 kDa) (Oertle et al., 2003) that is unable to cross the blood–spinal cord barrier. Based on pre-clinical reports, a pump and intrathecal catheter approach was used in 4 of 6 dosing regimens in the clinical trial (Hawryluk et al., 2008). This method circumvents the blood-spinal cord barrier, yet is not approved for use after acute SCI and presents a risk of serious infection (Aprili et al., 2009). An ideal delivery method would combine the safety of bolus injection with the efficacy of sustained release offered by catheter based approaches.

We have developed a drug delivery system that consists of drug-loaded poly(lactic-*co*-glycolic acid) (PLGA) nanoparticles embedded in a hydrogel of hyaluronan and methyl cellulose (HAMC). The nanoparticles attenuate the rate of drug release, while the hydrogel localizes the particles at the site of injection in the intrathecal space. The HAMC delivery platform is minimally invasive, biocompatible for at least 28 d in the intrathecal space (Baumann et al., 2010; Gupta et al., 2006; Kang et al., 2009), and has been used to deliver erythropoietin (Kang et al., 2009), fibroblast growth factor 2 (Kang et al., 2010), and vascular endothelial growth factor/angiopoietin-2 (Baker et al., 2010) in vivo. PLGA particle-loaded HAMC (composite HAMC) has been formulated to deliver a variety of small molecules and proteins over 28 d in vitro (Baumann et al., 2009b). The clinical interest in anti-NogoA and the demonstrated need for sustained release motivated us to investigate formulations of anti-NogoA in composite HAMC. The present work utilizes a new sandwich ELISA, which has been shown to detect only bioactive anti-NogoA, thereby allowing us to quickly screen PLGA nanoparticle formulations (Baumann et al., 2009a).

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A key challenge in encapsulating a biomolecule in PLGA particles is preserving activity during synthesis and release, because many proteins are susceptible to conformational damage caused by attendant environments. For example, Han et al. (2007) observed degradation and aggregation of recombinant human serum albumin after lyophilization, a problem that was only solved with the addition of sugar excipients. Sugars can stabilize proteins by satisfying the hydrogen bonding requirements of the protein after water sublimation (Carpenter et al., 1990). Proteins encapsulated within PLGA particles are also subject to damage from exposure to the aqueous–organic solvent interface during synthesis and the acidic environment which forms in PLGA particles as the polymer degrades over time (Bilati et al., 2005). The problem of organic solvent contact has been addressed by Lee et al. (2007) through the co-encapsulation of hyaluronan, which has been suggested to improve protein stability by creating a viscous microenvironment that slows the interaction between the protein and the interface. Co-encapsulated bases can neutralize the acidic environment inside PLGA particles, and were reported to stabilize encapsulated bovine serum albumin (Zhu et al., 2000). In both instances, additives can affect the release kinetics of the encapsulated protein. Water soluble additives can act as pore-forming agents (porogens), accelerating drug release by increasing the number and connectedness of pore networks (Dorati et al., 2010), while co-encapsulated bases slow acid-catalyzed PLGA degradation, retarding the rate of delivery (Ara et al., 2002). Strategies used to improve bioactivity must also be engineered not to change desired release kinetics.

We report the first sustained release formulation of anti-NogoA with the development of PLGA nanoparticle formulations which, when dispersed in HAMC, yield sustained release of bioactive anti-NogoA over 28 d. Formulations were designed based on a screen of excipients proposed to address three sources of bioactivity loss: lyophilization, organic solvent contact, and acidification of the PLGA microenvironment.

2. Materials and methods

2.1. Materials

The anti-NogoA mAb 11c7 was generously donated by Novartis (Basel, CH). Trehalose, $MgCO_3$, sodium dodecyl sulfate, poly(DL-lactic-*co*-glycolic acid) 50:50 of inherent viscosity 0.15–0.25 dL/g, and IgG from human serum of reagent grade were purchased from Sigma–Aldrich (Oakville, CA). Poly(vinyl alcohol), 6000 g/mol, was purchased from Polysciences Inc. (Warrington, USA). Sodium hyaluronate, 2600 kg/mol was purchased from Lifecore (Chaska, USA). Methyl cellulose, 300 kg/mol, was purchased from Shin-Etsu (Tokyo, Japan). Sodium hydroxide was purchased from EMD Chemicals (Gibbstown, USA). Pluronic F-127 was purchased from BASF (Mississauga, CA).

Artificial cerebrospinal fluid (aCSF) at pH of 7.4 was prepared as described by Gupta et al. (2006). HPLC grade dichloromethane (DCM), dimethyl sulfoxide (DMSO), and hydrochloric acid (HCl) were purchased from Caledon Labs (Georgetown, CA). Dulbecco's phosphate buffered saline (pH 7.4, 9.55 g/L) was purchased from Wisent Inc. (St-Bruno, CA). All buffers were prepared using water distilled and deionized using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus at 18 MΩ resistance (Millipore, Bedford, USA).

2.2. Nanoparticle processing and hydrogel preparation

Nanoparticles loaded with anti-NogoA were produced using a water/oil/water (w/o/w) double emulsion solvent evaporation technique, described elsewhere (Baumann et al., 2009b). Briefly, an inner aqueous phase of 178 μL aCSF containing 0.72 mg anti-NogoA and 1.36 mg IgG was mixed with an organic phase of 1.6 mL DCM, 80 mg PLGA and 0.8 mg Pluronic F-127. This mixture was sonicated using a Vibra-Cell (Sonics, Newtown, USA) on ice for 10 min at 26 W and 20 kHz to create the primary emulsion, which was subsequently mixed with the outer aqueous phase of 5.3 mL of 25 mg/mL PVA. The secondary emulsion was formed through sonication on ice for an additional 10 min at 39 W and 20 kHz. This double emulsion was then added to 53 mL of 25 mg/mL PVA and stirred gently for 20 h at room temperature. PLGA nanoparticles were isolated washed 4 times by ultracentrifugation (Beckman, Mississauga, CA), lyophilized (Labconco, Kansas City, USA), and stored at −20 °C until use. In syntheses with co-encapsulated trehalose, 25 mg trehalose was added to the inner aqueous phase of the emulsion in place of IgG. In the batches containing trehalose and hyaluronan, 25 mg trehalose and 1.8 mg hyaluronan were added to the inner aqueous phase in place of IgG. In the formulations which included base, 35 mg $CaCO_3$ or 6 mg $MgCO_3$ were added to the organic phase in addition to 25 mg trehalose and 1.8 mg hyaluronan in the inner aqueous phase.

HAMC hydrogels were prepared through the physical blending of hyaluronan and methyl cellulose in aCSF to achieve a final composition of 1 wt% 2600 kg/mol hyaluronan and 3 wt% 300 kg/mol methyl cellulose after addition of the PLGA nanoparticles. Methyl cellulose was first dispersed in the aCSF using a dual asymmetric centrifugal mixer (Flacktek Inc., Landrum, USA) and left to dissolve overnight at 4 °C, followed by hyaluronan which was dissolved in the same manner.

2.3. Particle characterization

Particle size was measured using dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Particle yield was defined as the total mass of particles produced divided by the initial mass of PLGA used, adjusted for protein content. Drug loading is the mass fraction of anti-NogoA in the particles and encapsulation efficiency is the measured protein loading of the particles divided by the theoretical maximum drug loading. To determine the total protein encapsulation efficiency, 1 mg nanoparticles were dissolved in 5 mL DMSO and added to 5 mL of 0.05 M NaOH containing 0.05 wt% SDS and analyzed using the total protein BCA assay according to the manufacturer's instructions (Thermo Scientific, Nepean, CA). To determine anti-NogoA encapsulation efficiency, 1 mg of particles was dissolved in 1 mL DCM for 1 h. The protein was then extracted into a liquid phase of 10.5 mL reagent diluent and analyzed using an anti-NogoA ELISA. This ELISA uses a fragment of NogoA that contains the sequence against which the capture antibody was raised, ensuring that only biologically active anti-NogoA was detected (Baumann et al., 2009a).

2.4. Drug release studies

Release profiles of anti-NogoA from each formulation were obtained by dispersing 10 mg of particles in 0.1 mL of concentrated HAMC in a 2 mL microcentrifuge tube (Axygen, Union City, USA) using a dual asymmetric centrifugal mixer at 3300 rpm for 4 min to produce a final composition of 8 wt% particles, 1 wt% hyaluronan, and 3 wt% methyl cellulose. The composite was then warmed to 37 °C for 10 min to induce gelation and 0.9 mL pre-warmed aCSF was added to the sample tubes. The supernatant was removed and replaced completely at 3 and 6 h, and 1, 3, 7, 14, 21, and 28 d. The protein content of the supernatant was determined by BCA assay and the bioactive 11c7 concentration by ELISA. The anti-NogoA remaining inside the particles was quantified after 28 d by dissolving the remaining particles in HAMC in 0.1 mL DCM and extracting

into 1 mL reagent diluent for protein quantification by BCA assay. Bioactive anti-NogoA was measured by ELISA.

2.5. Mathematical model

A mathematical model constructed in Matlab (MathWorks, Natick, USA) was used to quantitatively describe the effect of various processing parameters on the kinetics of anti-NogoA release. Based on the models developed by Faisant et al. (2002) and Raman et al. (2005), with minor modifications, release from composite HAMC was simulated in two parts: release from PLGA particles was simulated using a one-dimensional Fickian diffusion model in spherical coordinates and release from the HAMC hydrogel was simulated using a one-dimensional Fickian diffusion model in Cartesian coordinates.

In the governing equations and boundary conditions for release from PLGA nanoparticles given below, c is concentration, r is radial position inside the nanoparticles, $D(M_w)$ is diffusivity, and $f(r)$ is the initial anti-NogoA distribution inside the nanoparticles, which was taken as uniform.

Governing equation:

$$\frac{\partial c}{\partial t} = \frac{1}{r^2}\frac{\partial}{\partial r}\left(r^2 D(M_w)\frac{\partial c}{\partial r}\right)$$

Boundary conditions:

$$c(r)|_{t=0} = f(r) = 1$$
$$\left.\frac{\partial c}{\partial r}\right|_{r=0} = 0$$
$$C\big|_{r=R} = 0$$

The governing equations and boundary conditions for release from PLGA nanoparticles embedded in HAMC are:

Governing equation:

$$\frac{\partial c}{\partial t} = D(M_w)\frac{\partial^2 c}{\partial x^2}$$

Boundary conditions:

$$c(r)|_{t=0} = f(r) = 1$$
$$c|_{x=0} = c(t)$$
$$c|_{x=X} = 0$$

In which case x is position inside the HAMC hydrogel, $f(x)$ is the initial anti-NogoA distribution within the HAMC hydrogel, assumed to be uniform, and $c(t)$ is the predicted anti-NogoA concentration as a result of anti-NogoA released from nanoparticles alone.

The diffusivity term was treated similarly in both cases such that;

when fraction released < fraction available for burst release (F_{burst}),

$$D_{M_w} = D_0$$

and when fraction released > fraction available for burst release (F_{burst}),

$$D_{M_w} = ke^{k_{deg}t}$$

where D_0 is the initial diffusivity of anti-NogoA through the nanoparticles, k is a fit parameter representing the degree to which degradation influences diffusivity, and k_{deg} is the first-order degradation rate constant of PLGA.

2.6. Statistical analysis

All data are presented as mean ± standard deviation. To assess statistical differences between pairs of means, Student's t-tests were conducted and significance was assigned at $p < 0.05$ unless otherwise specified.

3. Results

3.1. Co-encapsulated trehalose improves the bioactivity of anti-NogoA and does not influence release kinetics

Excipients may be used to improve the stability of proteins encapsulated within PLGA particles (Bilati et al., 2005). Co-encapsulated trehalose improved the bioactivity of released anti-NogoA significantly at 1, 2, and 7 d compared to PLGA nanoparticles with no co-encapsulants (Fig. 1). Both release profiles were well described by a first-order degradation model wherein the initial bioactivity (F_0) of the trehalose formulation was found to be 100% compared to 55% for the control formulation. The first-order degradation term was similar for both cases at 0.010 h^{-1} and 0.012 h^{-1} for the trehalose and control formulations, respectively, as reported in Table 1. The model data indicate that trehalose protects anti-NogoA from denaturing during particle processing, but not during release. Co-encapsulated trehalose did not affect anti-NogoA release kinetics over 54 d compared to a control without any co-encapsulants (Fig. 2). In both cases, a burst of 10% was observed over the first 3 d, followed by release totaling 21% of the drug payload after 54 d.

3.2. Trehalose and hyaluronan together increase anti-NogoA release rate and bioactivity

Hyaluronan was added to the trehalose formulation to further improve protein bioactivity by increasing the viscosity inside the PLGA particles, minimizing protein–organic solvent interactions (Lee et al., 2007). When hyaluronan and trehalose were co-encapsulated, anti-NogoA was released at a faster rate over 54 d compared to formulations without hyaluronan. This release profile was characterized by a 22% burst release over the first 3 d, followed by a slower release rate up to 54 d, at which point 66% of the initial anti-NogoA was released (Fig. 2). As with the initial and trehalose-containing formulations, anti-NogoA release was well fit by model parameters, summarized in Table 2, with $R^2 \geq 0.95$ in all cases. Initial diffusivity (D_0) ranged from 2×10^{-17} to 2×10^{-16} cm^2/s. This is in close agreement with published diffusivity through 200 nm poly(lactic acid) particles (Budhian et al., 2008; Chorny et al., 2002; Rouzes et al., 2003), which are comparable in size to our 300 nm

Table 1
First-order bioactivity loss model parameters for the formulations presented in Figs. 1, 2 and 4.

$F = F_0 e^{-kt}$		
Excipient	F_0	k (h^{-1})
None	0.55	0.012
Trehalose (T)	1.00	0.010
T + hyaluronan (H)	1.00	0.011
T + H + $CaCO_3$	1.00	0.011
T + H + $MgCO_3$	1.00	0.017

Table 2
Anti-NogoA release model parameters for the formulations described in Figs. 2 and 4.

Excipient	F_{burst} (%)	D_0 (cm^2/s)	k	R^2
None	17	2×10^{-17}	0.012	0.96
Trehalose (T)	16	3×10^{-17}	0.005	0.98
T + hyaluronan (H)	21	2×10^{-16}	0.055	0.99
T + H + $CaCO_3$	19	2×10^{-17}	0.005	0.95
T + H + $MgCO_3$	11	3×10^{-17}	0.035	0.99

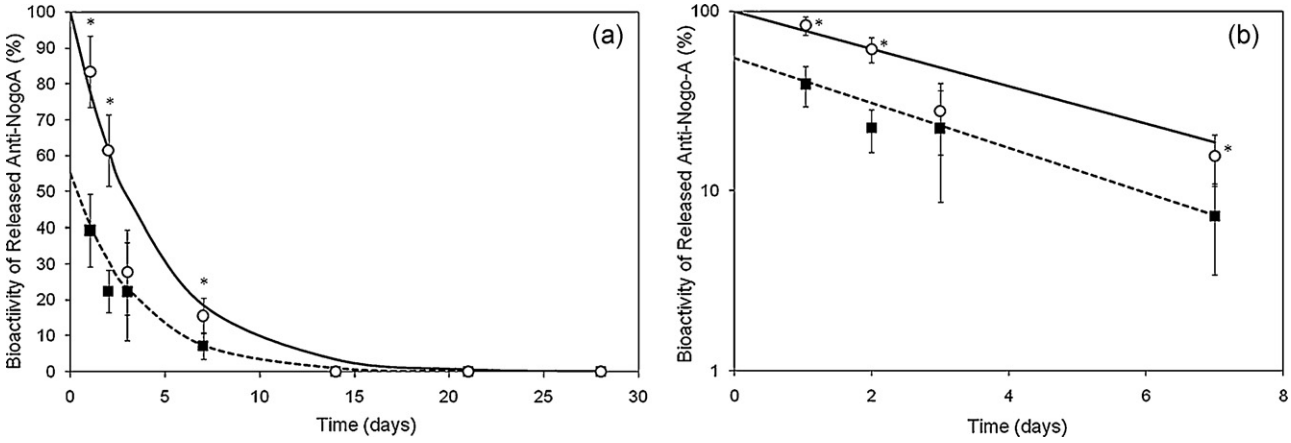

Fig. 1. Co-encapsulated trehalose significantly improves the initial bioactivity of released anti-NogoA. (a) The percentage of anti-NogoA that is bioactive during release is significantly higher ($p < 0.05$, $n = 3$) at 1, 2, and 7 days when (○) trehalose is co-encapsulated with anti-NogoA compared to a formulation with (■) no additives. At 14, 21, and 28 d, there was no measurable bioactivity for either formulation. A first-order bioactivity loss model was used to describe anti-NogoA bioactivity for (—) co-encapsulated trehalose and (- -) no excipients. (b) The first 7 d of data were plotted on a semi-log plot to demonstrate that the improvement to bioactivity is a result of increased initial bioactivity, rather than a change in the rate of bioactivity loss.

particles. Release profiles of formulations containing hyaluronan were fitted with the highest F_{burst}, D_0, and k values, providing evidence that this molecule is a porogen capable of increasing the number and connectedness of the pore network as it dissolves (Dorati et al., 2010). Greater porosity increases the fraction of anti-NogoA that is available for burst release and augments the rate of release because of the additional pathways available for escape. Interestingly, the high k parameter suggests that continuing pore formation due to polymer degradation over time is highest for the formulation containing trehalose and hyaluronan. This result also suggests that HA acts as a porogen because increased initial porosity allows for more and earlier water uptake, promoting PLGA hydrolysis and matrix degradation.

Encapsulation efficiency of the formulations was 97% for no additives, 89% for trehalose, and 43% for trehalose and hyaluronan. The reduced encapsulation efficiency may arise from the competitive interaction of anionic hyaluronan and anionic PLGA with anti-NogoA. It has been reported that ionic interactions between a protein and the free carboxylic end groups on PLGA are an important determinant of encapsulation efficiency (Yeo and Park, 2004).

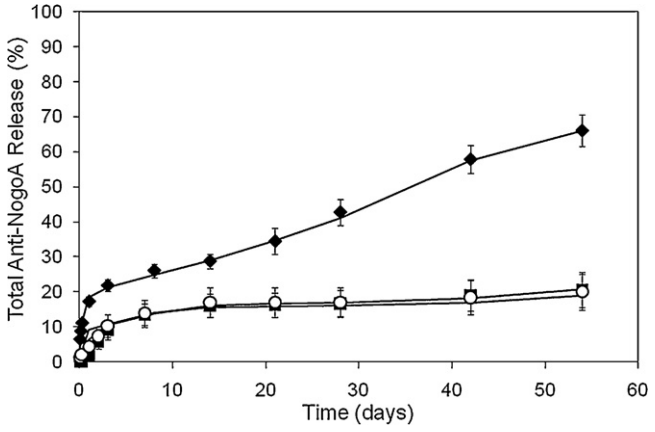

Fig. 2. Co-encapsulated trehalose does not influence anti-NogoA release kinetics, while hyaluronan and trehalose enhance sustained anti-NogoA delivery. When (○) trehalose was co-encapsulated in a formulation, a total anti-NogoA release profile was obtained similar to an (■) additive-free formulation. On the other hand, (♦) co-encapsulated trehalose and hyaluronan increased the burst amount and long-term release rate of anti-NogoA. All traces in this figure are simulations developed using the model parameters reported in Table 2.

We next explored whether the addition of hyaluronan had an impact on the bioactivity of anti-NogoA released from composite HAMC. When hyaluronan and trehalose were co-encapsulated with anti-NogoA, bioactivity was maintained at early time points as in the trehalose only formulation and significantly improved at 14, 21, and 28 d (Fig. 3). The first-order degradation term was 0.011 h^{-1} for the trehalose + hyaluronan formulation, as reported in Table 1.

3.3. Co-encapsulated bases reduce long-term release kinetics and do not affect anti-NogoA bioactivity at early release points

Seeking to further improve long-term anti-NogoA bioactivity, $MgCO_3$ or $CaCO_3$ were co-encapsulated in PLGA nanoparticles containing trehalose and hyaluronan. These bases were selected based on reports that they neutralize the acidic environment inside the PLGA particles over extended time periods and may therefore protect the encapsulated anti-NogoA from hydrolysis/denaturation. $MgCO_3$ was chosen based on results that demonstrate its ability to neutralize the acidic environment inside PLGA particles (Li and Schwendeman, 2005) and stabilize tetanus toxoid in PLGA particles (Jiang and Schwendeman, 2008). $CaCO_3$ was chosen because it is similar to $MgCO_3$ in terms of size and basicity, yet is less soluble and thus a poorer pore-forming agent. It was hypothesized that the release kinetics of anti-NogoA could be modulated through the selection of basic additives. It was predicted that the more soluble $MgCO_3$ would dissolve and create pores in the PLGA particles faster than $CaCO_3$, leading to more rapid release.

Incorporating $MgCO_3$ or $CaCO_3$ reduced the 3 d burst release from 22% to 7% and 15%, respectively (Fig. 4a), and yielded particles between 300 and 358 nm in diameter. Both co-encapsulated base formulations also showed significantly slower anti-NogoA release, suggesting the bases at least partially prevented acidification of the micro-environment inside the particles. Acid-catalyzed PLGA hydrolysis leads to matrix degradation and is responsible for a substantial portion of release from PLGA particles (Giteau et al., 2008). In this case the bases substantially reduced release up to 54 d. Compared to the 66% release after 54 d in the base-free formulation, only 19% and 23% were released in the same time frame for the $MgCO_3$ and $CaCO_3$ formulations, respectively. Anti-NogoA encapsulation efficiency approximately doubled when $MgCO_3$ or $CaCO_3$ were included in formulations containing trehalose and hyaluronan: 80% for $MgCO_3$ and 90% for $CaCO_3$, compared to 43% for trehalose and hyaluronan alone. This increase is likely attributed to the co-encapsulated bases accumulating in the aqueous phase

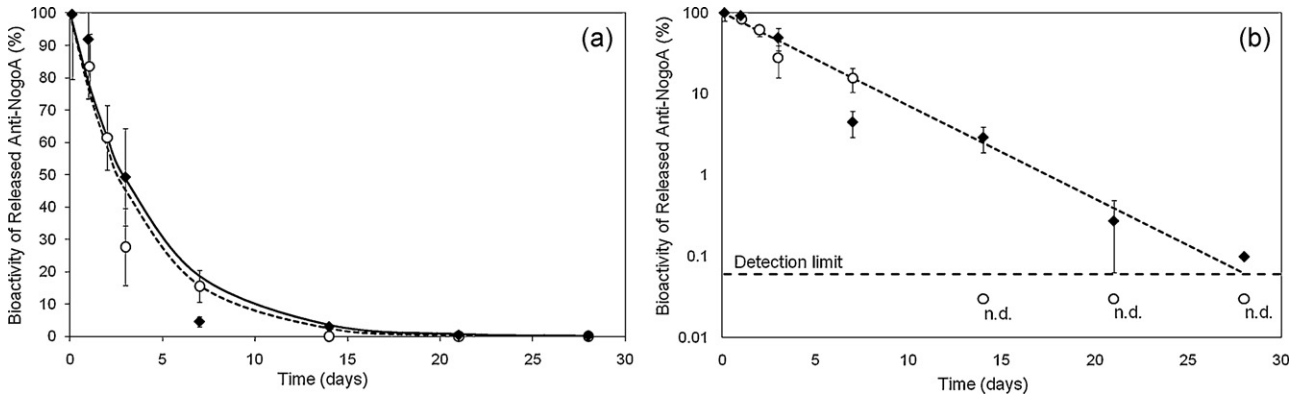

Fig. 3. Co-encapsulated hyaluronan with trehalose significant improves bioactivity of released anti-NogoA at late time points. (a) Anti-NogoA bioactivity is similar over the first 7 d comparing (○) co-encapsulated trehalose to (♦) co-encapsulated trehalose and hyaluronan, but the latter formulation has significantly higher bioactivity at 14, 21, and 28 d ($p < 0.05$, $n = 3$). First-order bioactivity loss models for (—) co-encapsulated trehalose and (- -) co-encapsulated hyaluronan were plotted. (b) The bioactivity data was plotted on a semi-log plot to illustrate the improvement to bioactivity garnered by (♦) co-encapsulating trehalose and hyaluronan. No bioactive anti-NogoA was detected in the trehalose formulation at 14 d or later.

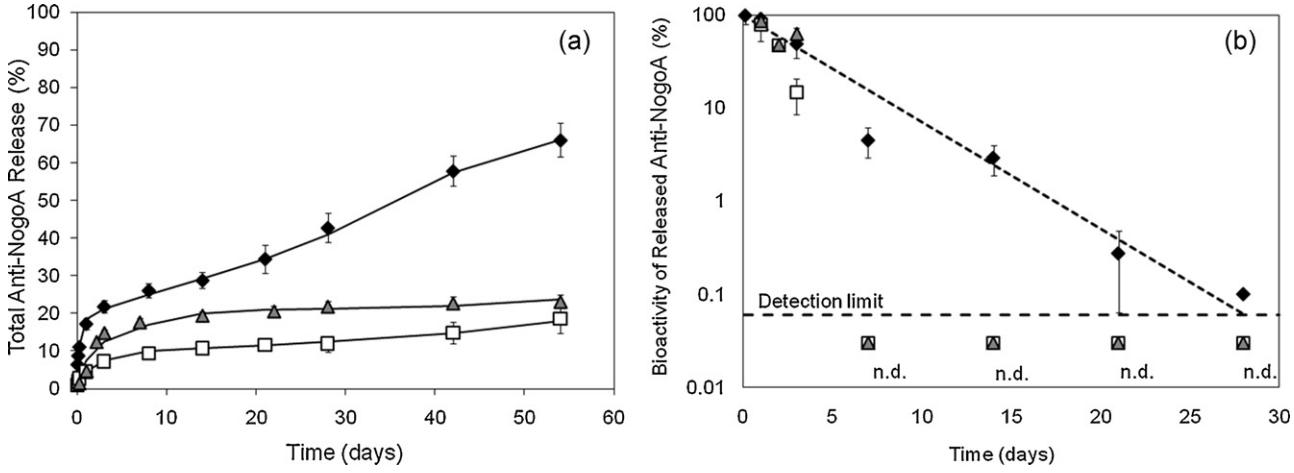

Fig. 4. Co-encapsulated bases reduce the release rate of anti-NogoA release such that bioactive anti-NogoA is undetectable after day 4. (a) When (▲) $CaCO_3$ or (□) $MgCO_3$ were co-encapsulated with the excipients trehalose and hyaluronan, the anti-NogoA total release profiles were dramatically reduced compared to (♦) co-encapsulated trehalose and hyaluronan. All traces in this figure are fits developed using the model parameters reported in Table 2. (b) A bioactivity semi-log plot demonstrates that trehalose + hyaluronan nanoparticle formulations with (▲) co-encapsulated $CaCO_3$ or (□) co-encapsulated $MgCO_3$ demonstrated similar bioactivity up to 3 d compared to a formulation with (♦) co-encapsulated trehalose and hyaluronan. There was no detectable bioactive anti-NogoA for the base-encapsulated formulations at from 7 d onward. Bioactive anti-NogoA was likely undetectable at later time points due to the reduced rate of release. A first-order bioactivity loss model is shown for the (- -) no base formulation.

rather than on the surface of the particles, due to their high solubility in water. This accumulation reduces the effect of the bases on the free carboxylic acid groups on the PLGA, allowing more ionic interactions between the PLGA and the anti-NogoA, which increases encapsulation efficiency (for a thorough review of PLGA encapsulation process, see (van der Walle et al., 2009).

Anti-NogoA bioactivity over the first 3 d of release was not influenced by the inclusion of co-encapsulated base in the trehalose plus hyaluronan formulation (Fig. 4b) and F_0 was found to be 100% in each case. Beginning at day 7, however, no additional bioactive anti-NogoA was detected. The first-order degradation term was similar for each formulation, being 0.011 h^{-1}, 0.017 h^{-1}, and 0.011 h^{-1} for the $CaCO_3$, $MgCO_3$, and no base formulations, respectively, as outlined in Table 1.

4. Discussion

Composite HAMC is a minimally invasive, injectable drug delivery system designed for localized and sustained release to the injured spinal cord where it has been shown to be biodegradable and biocompatible (Baumann et al., 2009b; Kang et al., 2009). Here, for the first time, we investigate a formulation for sustained release of bioactive anti-NogoA. Using composite HAMC, anti-NogoA was encapsulated in PLGA nanoparticles which were screened with three classes of excipients for their effect on anti-NogoA bioactivity, encapsulation efficiency, and release profile in vitro.

The double-emulsion synthesis of drug-loaded PLGA particles includes processes known to denature biomolecules, including: contact with organic solvents, high energy emulsification, and lyophilization (Bilati et al., 2005). Anti-NogoA encapsulated in our initial, excipient-free nanoparticle formulation illustrates this damage, suffering a 45% loss in bioactivity. Bioactivity was quantified using a sandwich ELISA that utilizes part of the NogoA extracellular domain as the capture antibody, thereby detecting only bioactive anti-NogoA (Baumann et al., 2009a). Trehalose is known to protect proteins during lyophilization (Wang, 2000) by providing a hydrogen bonding partner for the protein as water is sublimed (Carpenter et al., 1990). Co-encapsulation of trehalose preserved

all anti-NogoA bioactivity during processing (F_0 = 100%), suggesting that lyophilization was responsible for the 45% bioactivity loss in the initial formulation and leading us to reject our hypothesis that additional excipients would be required to mitigate sonication and DCM-related damage. Interestingly, co-encapsulated trehalose did not change the release kinetics of anti-NogoA, likely due to the small size of trehalose (M_w = 0.38 kDa) relative to anti-NogoA (M_w = 210 kDa) and correspondingly small and/or poorly interconnected pore network formed upon encapsulation. This result suggests that trehalose may be added to an optimized sustained release formulation to improve bioactivity without altering release kinetics. Although co-encapsulating trehalose resulted in the release of significantly more bioactive anti-NogoA up to 7 days, it did not slow the rate of anti-NogoA degradation inside PLGA, i.e. there was little change in the degradation term, k. The observed decline in released bioactive anti-NogoA to below the detection limit by day 14 suggested the need for a second class of excipient, one that stabilizes the protein during release. We additionally sought to extend release from a trehalose-containing formulation to 28 days.

Hyaluronan has been reported to preserve the bioactivity of encapsulated lysozyme in PLGA. We therefore asked whether co-encapsulating hyaluronan with trehalose would result in improved sustained release of bioactive anti-NogoA relative to trehalose alone. Co-encapsulating hyaluronan dramatically improved the sustained release profile and improved bioactivity up to 28 days. The larger hyaluronan (M_w = 2600 kDa) likely forms a larger pore network than trehalose upon dissolution that allows for faster and more complete diffusive anti-NogoA release. This mechanism is supported by the model fit parameters, which reveal that co-encapsulated hyaluronan increased the burst fraction (F_{burst} = 21% vs. F_{burst} = 16%), initial anti-NogoA diffusivity through the particles ($D_0 = 2 \times 10^{-16}$ vs. $D_0 = 3 \times 10^{-17}$ cm^2/s), and long-term, PLGA degradation controlled release (k = 0.055 vs. k = 0.005). These results suggest that co-encapsulating lower molar mass additives with higher molar mass therapeutics does not affect the release kinetics of the larger biomolecules. The sensitivity of anti-NogoA release to excipient size is consistent with the large body of literature on the effect of membrane pore size on solute diffusivity, which states that diffusivity is very sensitive to changes in pore size and that minimal solute diffusion is typically observed as the hydrodynamic radius of the solute approaches that of the pore (Deen, 1987). Future work will build on this formulation to further increase the bioactivity, especially after 21 days of release.

The long-term improvement in anti-NogoA bioactivity in the hyaluronan containing formulation may be due to reduced adsorption of anti-NogoA onto the surface of the polymer in the solid phase, a known problem that reduces protein stability in PLGA particles (Giteau et al., 2008). Carrier proteins such as bovine serum albumin have been included in particle formulations to reduce aggregation (Karal-Yilmaz et al., 2011), but foreign proteins are immunogenic. Use of hyaluronan may therefore be advantageous because it is native to the human body and does not typically trigger a foreign body response (Leach et al., 2003). In fact, hyaluronan has been shown to attenuate the inflammatory response in the central nervous system (Gupta et al., 2006). Adsorption can also lead to the formation of insoluble, inactive protein-PLGA aggregates which are not released until the PLGA matrix has completely degraded. The enhanced release and improved bioactivity of anti-NogoA in the hyaluronan formulation may stem from either or both of these adsorption related effects. Lee et al. (2007) previously suggested that hyaluronan-mediated improvement in protein stability was derived from the formation of a viscous aqueous phase that inhibits the interaction between the protein and the organic phase during double emulsion synthesis. Further study is required to distinguish between these potential mechanisms.

Weak bases comprise a third class of excipient, proposed to maintain bioactivity by slowing or preventing acidification of the PLGA microenvironment (Li and Schwendeman, 2005). This strategy has been used to stabilize bovine serum albumin (Zhu et al., 2000), basic fibroblast growth factor (Zhu et al., 2000), bone morphogenetic protein-2, (Zhu et al., 2000), and tissue plasminogen activator (Kang and Schwendeman, 2002). However, raising the pH within the matrix necessarily slows the rate of acid-catalyzed PLGA hydrolysis and attendant rate of pore network formation. Ara et al. (2002) previously reported this base-mediated reduction in PLGA degradation, which is in agreement with the attenuated release we observed after co-encapsulation with the bases $MgCO_3$ or $CaCO_3$ in the present work. Interestingly, the base neither altered anti-NogoA bioactivity at early time points, which was expected since the acidic environment inside PLGA particles takes several days to form (Li and Schwendeman, 2005), nor improved bioactivity at later time points, suggesting that anti-NogoA activity is not lost via pH induced denaturing. In the future, it may be possible to tune the concentration of base within the nanoparticle to balance pH moderation and sustained release, which may yield a benefit to long-term anti-NogoA bioactivity.

The rate of anti-NogoA release from composite HAMC compares favorably to that obtained from many successful preclinical reports of functional recovery, with the formulation containing trehalose and hyaluronan preferred as it provides sustained bioactive release up to 28 days. Composite HAMC with co-encapsulated trehalose and hyaluronan should deliver up to 60 μg of anti-NogoA per rat over 28 d based on a 10 μL injected volume, a particle loading of 200 mg/mL, and an anti-NogoA loading of 30 μg/mg of PLGA. Adjusting for the smaller distribution volume, Wu et al. (2010) delivered 2.7 μg of anti-NogoA over 28 d in a rat model and observed significant functional recovery. Normalizing the dosing in a macaque monkey based on the ratio of cerebrospinal fluid volume against the rat, 30 mL vs 200 μL (Matin and Goodwin, 1971), Freund et al. (2006) delivered 4.7 μg of anti-NogoA over 14 d and observed improved recovery of manual dexterity and sprouting of corticospinal axons. Based on these reports, we are encouraged by the pharmacologically relevant anti-NogoA dose, sustained release, bioactivity retention, and biocompatibility of composite HAMC. This provides us with an excellent framework with which to study the anti-NogoA delivery from composite HAMC in a rat model of SCI.

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