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Controlled delivery of VEGF via modulation of alginate microparticle ionic crosslinking

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Abstract

Clinical application of therapeutic angiogenesis is hampered by a lack of viable systems that demonstrate controlled, sustained release of vascular endothelial growth factor (VEGF). Alginate has emerged as a popular material for VEGF delivery; however most alginate-based systems offer limited means to control the rate of VEGF release beyond reducing the VEGF:alginate ratio to suboptimal efficiency. This study describes methods to control the release of VEGF from small (<10 μm mean diameter) alginate microparticles via the use of different ionic crosslinkers. Crosslinking with Zn^{2+} versus Ca^{2+} reduced VEGF diffusional release and the combination of discrete populations of either Zn^{2+} - or Ca^{2+} -crosslinked particles allowed for control over the sustained release profiles for VEGF. The particle preparations were non-toxic and VEGF was bioactive after release. These results demonstrate that ionic modulation of alginate crosslinking is a viable strategy for controlling release of VEGF while retaining the high protein:polymer ratio that makes alginate an attractive carrier for delivery of protein therapeutics.

Keywords

VEGF; Protein drug delivery; Therapeutic angiogenesis; Controlled release; Microparticles

1. Introduction

Peripheral vascular disease, resulting from atherosclerosis, is a major cause of mortality and morbidity worldwide through induction of critical limb ischemia (CLI) [1,2]. Therapeutic revascularization via delivery of angiogenic proteins, such as vascular endothelial growth factor (VEGF), has been identified as a key strategy for treatment of CLI in numerous animal models and small clinical studies [3–6]. VEGF is a ~45 kDa angiogenic protein that plays a critical role in both physiologic and pathologic vascular development [7]. Its use as a potential treatment for peripheral and myocardial vascular disease is well established [8], but clinical trials investigating the effect of bolus injections of VEGF have largely failed to show efficacy [9,10], likely because IV administration of VEGF is limited by its short *in vivo* half life (~30 min) and overall dose is limited by off-target site toxicity issues [11,12]. Interestingly, recent data indicates that VEGF mono-therapy can be a successful means of angiogenic induction, but that control over delivery rate and total dose are essential for production of normally functioning vascular networks [13]. Unfortunately, despite numerous studies that have examined sustained release approaches [3,5,14–25], delivery of VEGF at a high VEGF:vehicle ratio, such that therapeutic concentrations of VEGF can be achieved from non-toxic levels of

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delivery vehicle, is difficult to realize. However, one vehicle that has demonstrated the capacity to overcome this limitation is alginate [15–17].

Alginate, a linear copolymer derived from various species of kelp, is able to be crosslinked in an aqueous environment and has been shown to increase VEGF stability and bioactivity [17, 26]. In previous studies, VEGF has been delivered from macroscopic alginate gels or microbeads, the latter with diameters ranging from hundreds of microns to a millimeter or greater. We recently reported on the effectiveness of much smaller particles, with an average diameter of $\sim 10\ \mu\text{m}$, that were crosslinked by CaCl_2 and ZnCl_2 [27]; these particles provide sustained VEGF delivery and *in vivo* efficacy in association with endothelial cell transplantation for treatment of ischemia. The present study builds on these observations by examining the relationship of controlled VEGF release from small alginate microparticles to the crosslinking ion used.

Crosslinking of alginate occurs when its constituent monomers, mannuronic acid (M) and guluronic acid (G), are formed into poly-M, poly-G, and alternating MG blocks in the presence of any of numerous divalent or trivalent cations [28]. Ca^{2+} is, by far, the most widely used crosslinking ion; Ca^{2+} -crosslinked alginate is considered to be clinically safe [29]. Sr^{2+} - and Ba^{2+} -salts are also commonly considered to have low toxicity [28], and have been examined as crosslinkers for alginate microbeads and gels [30]. Zn^{2+} is also capable of crosslinking alginate, but is poorly suited as a crosslinking ion for pancreatic islet encapsulation, a common application for large alginate microbeads, due to sensitivity of beta cells to zinc [31]. Therefore, Zn^{2+} has scarcely been investigated as a crosslinker for alginate in protein delivery applications. However, zinc is capable of crosslinking alginate less specifically than other ions [32], forming GG, MG, and MM linkages whereas Ca^{2+} and Sr^{2+} form predominantly GG linkages and Ba^{2+} is incapable of linking M and G blocks together [30].

We hypothesized that the use of zinc, via increased crosslinking, could produce a drastically different VEGF release profile than those of particles crosslinked with other ions [33,34], especially when paired with an alginate possessing a high percentage of MG and MM blocks. Alginate derived from *Macrocystis pyrifera* is composed of a relatively high proportion of MM and MG blocks (84% combined), and so it was chosen for this study instead of other commercially available alginates with high-M content derived from *Laminaria hyperborea* (leaf) (74%) and *Laminaria digitata* (75%) [35]. The objective of this report was to examine the effect of crosslinking small alginate microparticles with Ba^{2+} , Ca^{2+} , Sr^{2+} , and Zn^{2+} , and combinations thereof, on release of VEGF and particle cytotoxicity. We further hypothesized that combining populations of particles with different release profiles would provide a means of controllable, sustained VEGF release that is typically not possible with alginate microparticle systems.

2. Materials and methods

2.1. Materials

Alginate from *M. pyrifera* (viscosity of ~ 250 cps (2% solution at $25\ ^\circ\text{C}$), ~ 50 kDa) was obtained from Sigma (St. Louis, MO, USA) and subsequently purified of endotoxins based on the procedure of Klock et al.[36], with confirmation using an LAL QCL-1000 kit (Lonza, Walkersville, MD, USA). VEGF- A_{165} (VEGF) was a generous gift of the National Cancer Institute and received lyophilized 1:50 with bovine serum albumin (ultrapure, $>98\%$ albumin). Human VEGF DuoSet enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, USA). EGM-2 medium was procured from Lonza (Walkersville, MD, USA). Cytodex 3 microcarriers were acquired from GE Healthcare (Chalfont St. Giles, UK). Fibrinogen, aprotinin, thrombin, hydroxypropylmethylcellulose (HPMC) and MTT reagent were all purchased from Sigma (St. Louis, MO, USA) and used

without further purification. Human umbilical vein endothelial cells (HUVEC) were a generous gift of Dr. Jordan Pober (Yale University).

2.2. Methods

2.2.1. Preparation of alginate microparticles—Alginate microparticles were prepared essentially as previously described [27] using an emulsification technique based on the method of Zheng et al. [37], with several modifications. Alginate and HPMC were co-dissolved in ultrapure H₂O to a final concentration of slightly greater than 20 mg/ml (9:1 alginate:HPMC). BSA:VEGF (50:1) was then dissolved at 1 mg/ml (20 µg VEGF/ml) and added to the alginate solution at a volume to make the final alginate:HPMC concentration 20 mg/ml. The resulting solution was subsequently added dropwise to iso-octane+5% (v/v) Span 80 while homogenizing at 17,500 rpm. 30% (v/v) aqueous Tween 80 was then added dropwise to the emulsion, resulting in an acceptable HLB value. The emulsion was mixed for 3 min, followed by the dropwise addition of an ionic crosslinking solution at total concentrations ranging from 90 mM to 700 mM. After a further 3 min of mixing, 2-propanol was added and the particles were allowed to cure for 3 min. Particles were then centrifuged at 4,000 rpm for 1 min, the supernatant was removed and particles were subsequently washed 2× for 8 min in 2-propanol. The particles were then air-dried, resuspended in ultrapure H₂O, and lyophilized. Based on preliminary data, Zn²⁺ crosslinked particles were then soaked in M199 medium for 2 h at 4 °C, the medium was filtered and the particles were lyophilized again overnight. Particles were stored at −20 °C until use.

2.2.2. Microparticle characterization—Microparticle morphology was evaluated via brightfield (Olympus IX71) and scanning electron microscopy (EM) (Philips XL series ESEM). For EM, samples were mounted to aluminum stubs with double-sided carbon tape and sputter-coated (Cressington Sputtercoater 108auto) with gold prior to viewing. Particle size was determined in aqueous solution using a Beckman Coulter Multisizer 3. Encapsulation efficiency was determined via an extraction method. Particles were incubated at 1 mg/ml in PBS at 37 °C under intense agitation for 12 h. Protein was extracted into the aqueous supernatant via centrifugation and quantified using a BCA assay. The extraction procedure was performed a total of 3× for each particle type.

2.2.3. VEGF release—VEGF release profiles were obtained by incubating particles at 3 mg/ml in 24 well plates at 37 °C with a 5% CO₂ atmosphere in triplicate. Particles were pooled from 3 different batches and plates were constantly agitated using an undulating platform. All VEGF concentrations were determined by ELISA and the release medium was 20% FBS-M199, with l-glutamine and penicillin/streptomycin supplements (complete M199).

To investigate the effect of crosslinking ion and concentration on the mechanism of VEGF release from small alginate microparticles, a semi-empirical model, known as the power law [38], was used:

$$M_t/M_\infty = kt^n \quad (1)$$

where M_t is the mass of drug released at a time t , M_∞ is the total mass of released drug, k is a constant incorporating morphologic characteristics of the delivery system, and n is the release exponent, provided that t is limited to times where $M_t/M_\infty < 0.6$. This model has been shown to correlate well for release of an agent from swellable hydrogels. For spherical release systems, an n value of 0.43 is indicative of a Fickian diffusion mechanism, a value between 0.43 and 0.85 indicates anomalous transport controlled by both diffusion and swelling, a value of 0.85 represents case-II transport (swelling controlled drug release), and values >0.85 indicate super case-II transport [39].

2.2.4. Particle cytotoxicity—HUVEC were routinely grown in complete M199 medium with endothelial cell growth supplement (ECGS). HUVEC before passage 6 were used for all experiments. Particle cytotoxicity to HUVEC was evaluated via direct addition of particles to cells in culture. HUVEC were seeded into gelatin-coated 96-well plates at 10,000 cells/well in EGM-2 and allowed to adhere overnight. Medium was then aspirated and immediately replaced with fresh EGM-2 containing the desired concentration of microparticles and allowed to incubate for 48 h without changing medium before determination of cell survival by the MTT assay.

2.2.5. VEGF bioactivity—VEGF bioactivity was determined by a sprouting assay. Sprouts of HUVEC from microcarriers in fibrin clots following exposure to released VEGF from each particle type were counted following clot fixation overnight in 4% paraformaldehyde. Microparticles were incubated in EGM-2 without growth factors for a continuous period of 1, 5, 10, or 15 days. VEGF values were measured by ELISA and subsequently HUVEC were cultured on Cytodex 3 microcarriers overnight and suspended within fibrin clots in 24-well plates with EGM-2 with growth factors, but without VEGF, as previously reported [40]. Release supernatant was then added to wells to make an equivalent final VEGF concentration of 10 ng/ml. Native, non-encapsulated VEGF at 10 ng/ml was used as the 100% bioactivity benchmark, and wells with medium only (no VEGF) were employed as the negative control. An average of 106 microcarriers was evaluated per condition.

2.2.6. Data presentation and statistical analyses—Unless indicated, data are represented as mean±standard error of the mean. Statistical significance was determined using a student's *T*-test with a 95% confidence interval, unless otherwise noted. Statistical calculations, non-linear curve fits, EC₅₀ determinations and linear regressions were performed using a GraphPad Prism software.

3. Results

3.1. Particle morphology and VEGF encapsulation efficiency

Alginate microparticles crosslinked by CaCl₂, ZnCl₂, and SrCl₂ exhibited spherical morphology in aqueous solution and swelled when placed in aqueous medium (Fig. 1a–f).

Particles crosslinked with BaCl₂ also swelled in suspension, but appeared less spherical in nature (Fig. 1g–h). Swelling for all particle types occurred rapidly, and was essentially complete within 20 min of suspension in release medium (data not shown). Furthermore, particle diameters for all crosslinkers after 20 min in suspension were found to be statistically similar to those for the same particle populations after 2 weeks incubation, including seven changes of release medium, indicating high morphological stability for all formulations examined (not shown). CaCl₂-crosslinked particles had statistically larger diameters than those crosslinked by other ions (Fig. 2a), but the values are too close to be practically considered different. No practical dependence of particle diameter on concentration of either ZnCl₂ or CaCl₂ was detected (Fig. 2b).

CaCl₂- and SrCl₂-crosslinked particles entrapped VEGF most effectively, while ZnCl₂-crosslinked particles had substantially lower encapsulation efficiency than other formulations (Fig. 2c). However, ZnCl₂-crosslinked particles released ~100% of their payload, while release from all other particles measured was significantly less (Fig. 2c). BaCl₂-crosslinked particles displayed the poorest VEGF encapsulation, further indicating the potential unsuitability of Ba²⁺ as a crosslinking ion in this application. VEGF encapsulation efficiency was not significantly affected by crosslinking ion concentration for Ca²⁺ and Zn²⁺ (Fig. 2d).

3.2. Cytotoxicity

Cytotoxicity of alginate microparticles was evaluated *in vitro* by direct addition to HUVEC. Initially, ZnCl₂-crosslinked particles were prepared without a post-emulsification wash step, as described in Methods. These unwashed particles (EC₅₀ = 795 µg/ml), as well as BaCl₂-crosslinked particles (EC₅₀ = 78 µg/ml), significantly reduced HUVEC viability, even at low concentrations (Fig. 3a). By comparison, SrCl₂-crosslinked particles were better tolerated at low concentrations, although the calculated EC₅₀ of 817 µg/ml was not significantly greater than the EC₅₀ value obtained for unwashed ZnCl₂-crosslinked particles. CaCl₂-crosslinked particles caused little reduction in cell viability until supersaturating particle concentrations were employed (EC₅₀ = 3091 µg/ml) (Fig. 3a). We hypothesized that Zn²⁺-induced cytotoxicity might be the result of a burst release of Zn²⁺ into the medium, as large, rapid increases in Zn²⁺ concentration can result in detrimental effects to cells and tissues [41]. Addition of a brief washing step resulted in a marked reduction in 700 mM ZnCl₂-crosslinked particle-induced cytotoxicity (EC₅₀ = 1230 µg/ml) (Fig. 3b). A similar reduction in toxicity was seen when using particles crosslinked with a 90 mM ZnCl₂ solution (EC₅₀ = 1843 µg/ml) (Fig. 3b). Also, the counter ion present in the Zn²⁺ salt used for crosslinking had an effect on cytotoxicity, with SO₄²⁻ producing a lower toxicity at higher concentrations (EC₅₀ = 2234 µg/ml) while use of acetate proved to be significantly more toxic (EC₅₀ = 329 µg/ml) (Fig. 3c). The wash step resulted in less than a 30 ng VEGF/mg particle loss (or 3% of the theoretical maximum loading), as measured by ELISA. When an identical washing procedure was applied to all other particle populations, significantly higher VEGF losses were recorded, and so this wash step was limited to Zn-crosslinked particles. Finally, we also observed an increase in cell viability relative to control at low ZnCl₂ concentrations; this was likely due to the anti-apoptotic effect of Zn²⁺ ions on endothelial cells [42].

3.3. Release of VEGF

Choice of crosslinking ion had a substantial impact on the VEGF release profile from alginate microparticles *in vitro*. VEGF release from BaCl₂- and SrCl₂-crosslinked particles followed a similar pattern, with a dramatic burst release and greater mass released corresponding to a lower crosslinker concentration (Fig. 4a,b). Release of VEGF from CaCl₂- and ZnCl₂-crosslinked particles exhibited a different trend, with a higher mass release facilitated by copious amounts of crosslinking ion (Fig. 4c,d). Furthermore, ZnCl₂-crosslinked particles displayed a significantly prolonged release of VEGF compared to particles formed with other ions. This profile of extended VEGF release was retained when another Zn²⁺ salt, zinc acetate, was used as the crosslinking ion (Fig. 4e), but not for ZnSO₄ (Fig. 4f), suggesting that ionic dissociation in solution plays a role in the availability of divalent cations for crosslinking in alginate-containing emulsions.

The effect of crosslinker modulation on the release mechanism of VEGF from small alginate microparticles was investigated using the power law expression (Eq. (1)). Based on this model, release of VEGF from 700 mM CaCl₂-crosslinked particles was anomalous (Fig. 5a,c), suggesting that both diffusion and particle swelling are important in determining rate of release. For ZnCl₂-crosslinked particles, the same analysis indicated a super class-II transport mechanism (Fig. 5b,c). It should be noted that alginate release systems are known to often exhibit a lag phase of release at very early time points [33]. In the case of ZnCl₂-crosslinked particles in this study, the lag phase appears to be not only present, but extended significantly compared to that of CaCl₂-crosslinked particles. Particles crosslinked by BaCl₂ and SrCl₂, as well as by lower concentrations of CaCl₂, exhibited an extreme burst release of VEGF within the first several hours of incubation in the release medium, and so were excluded from this analysis.

3.4. Controlled release of bioactive VEGF

Given the nature of the VEGF release profiles for ZnCl_2 - and CaCl_2 -crosslinked particles, we hypothesized that combining populations of particles crosslinked by each of these ions could yield a method for controlling VEGF release from alginate. By combining particles at various ratios, we observed that release of VEGF depended on ZnCl_2 : CaCl_2 -crosslinked particle ratio (Fig. 6a). Combining both ZnCl_2 and CaCl_2 into the same crosslinking solution, at various concentration ratios, did not have the same effect (Fig. 6b). The cytotoxicity of a 1:1 ZnCl_2 / CaCl_2 particle population was found to be nearly identical to that of a pure population of CaCl_2 -crosslinked particles ($\text{EC}_{50} = 3967 \mu\text{g/ml}$) (Fig. 6c), which have the most favorable biocompatibility characteristics of all alginate particles studied. Finally, we examined the bioactivity of VEGF encapsulated in particles crosslinked by ZnCl_2 and CaCl_2 , as well as mixed populations of such particles, using a HUVEC sprouting assay. Between formulations, no significant differences in bioactivity were observed out to 15 days post-encapsulation (Fig. 6d). Furthermore, in this *in vitro* assay, encapsulated VEGF bioactivity was not significantly different from that of native VEGF (positive control) for up to 10d for all formulations.

4. Discussion

Alginate is an effective means for encapsulation and delivery of bioactive VEGF; however, in prior reports, control over rate and duration of delivery was limited. Aside from modification of protein loading or alginate concentration, a number of methods have been proposed for modification of protein release from alginate micro-particles. Most notably, addition of a polycation layer or other coating as an additional diffusion barrier has been applied in a number of studies [43–46]. However, polycations are typically toxic [47] and can exacerbate the foreign body response to implanted alginate beads [48]. Our results support an alternative method for control of VEGF release: ionic modulation of alginate crosslinking allows control of release from small alginate microparticles without imparting toxicity.

Particle size was largely unaffected by the crosslinking ion used in particle formulation (Fig. 2a). Likewise, particle morphology was similar for all formulations except for those particles crosslinked with BaCl_2 (Fig. 1g,h). Ba^{2+} has been used in the crosslinking of large alginate beads for cell encapsulation [49–51]; it can induce formation of more stable capsules than those crosslinked by Ca^{2+} for certain alginate compositions [30,52]. However, Ba^{2+} was determined to be an undesirable crosslinking ion for this application due to the low encapsulation efficiency (Fig. 2c) and poor sustained release characteristics of BaCl_2 -crosslinked particles (Fig. 4a). This result is likely due to the alginate selected for this study; Ba^{2+} has been reported to have low affinity to alginate from *M. pyrifera* [53] based on its relative dearth of GG blocks (16%). As previously noted, alginate from *M. pyrifera* was selected for this study based on the likelihood of improved crosslinking with Zn^{2+} . Additionally, preliminary data indicated a lack of sustained VEGF release from Ca^{2+} -, Sr^{2+} -, and Ba^{2+} -crosslinked alginate derived from *L. hyperborea* (stem) (57% GG Blocks) (FMC Biopolymer, data not shown).

Surprisingly, Sr^{2+} -crosslinked particles were spherical and retained their morphological stability over the duration of VEGF release (not shown). Sr^{2+} is also thought to be incapable of forming MG linkages [30], instead forming only GG crosslinks. We believe that it forms less stable particles with the alginate used in this study due to the low GG block content of alginate from *M. pyrifera* (~18%) [35]. Sr^{2+} -crosslinked particles also show poor sustained release of VEGF (Fig. 4b), but VEGF encapsulation efficiency in Sr^{2+} -crosslinked particles was much higher than that of particles crosslinked by Ba^{2+} (Fig. 2c), indicating less competition between Sr^{2+} ions and VEGF for alginate binding sites. The competitive nature of crosslinking ion and VEGF for alginate binding sites is confirmed by the trend of increasing total VEGF release with decreased ion concentration for both Sr^{2+} and Ba^{2+} (Fig. 4a,b). Overall, these data indicate that enhanced MM or MG linkage formation leads to decreased VEGF entrapment,

and therefore that the electrostatically-based affinity interaction between VEGF and alginate is mediated predominantly by M-containing blocks in this system.

VEGF release after crosslinking with Ca^{2+} and Zn^{2+} ions revealed a different trend with ion concentration. While VEGF release decreased with increasing ion concentration at lower overall concentrations for both Zn^{2+} and Ca^{2+} , as was also seen with Sr^{2+} and Ba^{2+} , this trend was broken when particles were crosslinked by either 700 mM CaCl_2 or 700 mM ZnCl_2 (Fig. 4c,d). Notably, at 700 mM, Ca^{2+} - and Zn^{2+} -crosslinked particles released a significantly higher percentage of their payloads than did Ba^{2+} - and Sr^{2+} -crosslinked particles (Fig. 2c), indicative of a charge-shielding effect [54]. This leads to more complete crosslinking with these two ions, especially Zn^{2+} , as ZnCl_2 -crosslinked particles released nearly all of their payloads. Interestingly, the concentrations required to overcome the trend of increasing ion concentration leading to reduced VEGF encapsulation in this emulsification system are much higher than the crosslinking ion concentrations used for more traditional gelation of alginate microbeads in aqueous media [30]. This is likely due to the varying exposure times to ionic solution between conventional external gelation and external gelation during emulsification. Crosslinking with a 700 mM solution, which brings the overall aqueous ionic concentration to ~350 mM, may be necessary to instantaneously saturate the available crosslinking sites during emulsification. The concentration ranges used in this study were based on preliminary experiments that showed lower concentrations did not reproducibly induce stable alginate gelation while higher concentrations tended to induce increased cytotoxic effects.

A key difference between VEGF release from alginate crosslinked with Zn^{2+} and Ca^{2+} was revealed when the power law model was fit to release data (Fig. 5). Values of the release exponent for 700 mM CaCl_2 - and 700 mM ZnCl_2 -crosslinked particles indicate a different mechanism of release between these two particle populations. However, this difference can be partially accounted for by the prolonged lag phase of release observed with ZnCl_2 -crosslinked particles; at later times, VEGF release from these particles appears to be governed predominately by diffusion. This lag effect has been noted before for Zn^{2+} -crosslinked alginate [33], although it was previously attributed in part to an interaction between zinc and insulin, which was encapsulated in the earlier study. Another report noted no significant difference on the diffusion of a small molecule, acetaminophen, from alginate gels crosslinked with either Ca^{2+} or Zn^{2+} [32], but a decrease in permeability was observed to be associated with more complete crosslinking, such as that provided by Zn^{2+} . The apparent predominance of diffusion on release from Ca^{2+} - and Zn^{2+} -crosslinked alginate observed for VEGF in this study is consistent with results noted by Chan et al. [34]. However, this same study also described an inability to form spherical particles with Zn^{2+} as the sole crosslinking ion. The results reported here might be different due to the use, in our study, of substantially lower concentrations of crosslinker, different Zn^{2+} counterion (Cl_2 vs. SO_4), alginate type (*M. pyrifera* vs. *L. digitata*), or other formulation parameters. Specifically, we observed that at higher Zn^{2+} concentrations (>1 M), substantial particle agglomeration occurred (data not shown). The release data for Zn^{2+} -crosslinked particles in this study is also consistent with the findings of Pillay et al. [55], indicative that ionic crosslinker modulation to control release is not a VEGF-specific phenomenon, and may be applied more broadly for protein delivery from alginate.

We hypothesized that combining populations of particles would enable us to manipulate the VEGF release profile from small alginate microparticles. Indeed, several intermediate profiles were observed when combining different ratios of 700 mM CaCl_2 - and 700 mM ZnCl_2 -crosslinked particles (Fig. 6a), providing a degree of controlled release that is typically unobtainable for alginate-based systems, which are generally manipulated via different protein loadings or alginate concentrations. Reduction of protein loading is a problem with these systems, as high protein:polymer is a primary benefit of using this system [28]. Not surprisingly, combining CaCl_2 and ZnCl_2 in the same crosslinking solution at various

concentration ratios did not have a similar effect (Fig. 6b). Due to the high MG and MM block content of the alginate used for this study, Zn^{2+} crosslinking was expected to dominate based on its affinity for these blocks. The data show that incorporating CaCl_2 as a simultaneous crosslinker may prevent more extensive crosslinking by ZnCl_2 by restricting matrix mobility through GG linkages, which are known to be more mechanically stiff than MM or MG linkages [30]. However, this approach to controlling VEGF delivery may still be of some use [34], as nearly linear release profiles were observed for VEGF over 7 days of release (Fig. 6b). It is notable that overall release of VEGF was significantly decreased using this type of ionic modulation.

The cytotoxicity of Zn^{2+} -crosslinked alginate microparticles was significantly reduced by washing away the initial burst release of Zn^{2+} ions [41] (Fig. 3a,b). Also expectedly, Ca^{2+} -crosslinked particles were the least cytotoxic, as Ca-alginate is generally considered to be clinically safe [28,29]. Interestingly, a 1:1 mixture of Ca^{2+} - and Zn^{2+} -crosslinked particles displayed nearly identical cytotoxicity to particles crosslinked by Ca^{2+} alone (Fig. 6c). The validity of this finding is further supported in the literature [56] and by our previous demonstration of the *in vivo* therapeutic utility of such particles in a preclinical model of hindlimb ischemia [27]. Furthermore, mixed populations of particles crosslinked by Zn^{2+} and Ca^{2+} had statistically similar VEGF bioactivity profiles in an *in vitro* sprouting assay (Fig. 6d), indicating that neither choice of crosslinking ion nor relative ratio of particle populations substantially affects VEGF bioactivity for this formulation process. More importantly, VEGF bioactivity was retained at or near levels of native VEGF for at least 15 days. These results are far superior to those seen with a different delivery vehicle, poly(lactic-co glycolic acid), in the same assay [27]. In addition, we have recently shown that a 1:1 mixture of Zn^{2+} - and Ca^{2+} -crosslinked particles release bioactive VEGF *in vivo* [27]. Taken together, these data suggest that mixing populations of particles crosslinked by Zn^{2+} and Ca^{2+} represents a viable approach for tunable, sustained release of VEGF from small alginate microparticles.

5. Conclusion

We developed new methods for the controlled, sustained release of VEGF from small alginate microparticles. Prior studies have encountered an inability to tune VEGF release from alginate-based systems; we overcame this limitation by mixing populations of particles crosslinked by different divalent cations, Zn^{2+} and Ca^{2+} . The cytotoxicity and bioactivity of encapsulated VEGF in these particles was unaffected by this strategy, retaining the primary advantage of small alginate microparticles for VEGF delivery, namely the high VEGF:vehicle ratio needed for many clinical applications.

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Fig. 1. Morphology of small alginate microparticles. Scanning electron micrographs (a,c,e,g) and brightfield microscopy images (b,d,f,h) of alginate microparticles crosslinked by 700 mM CaCl_2 (a,b), 700 mM ZnCl_2 (c,d), 700 mM SrCl_2 (e,f), and 700 mM BaCl_2 (g,h). Scale bar=50 μm .

Fig. 2.

Effect of crosslinking ion on microparticle size and encapsulation efficiency. Mean particle diameter was relatively unaffected by species of crosslinking ion (a), although Ca^{2+} -crosslinked particles displayed statistically larger average diameters. Particle diameter was also essentially unchanged by variation of Ca^{2+} (squares) and Zn^{2+} (triangles) concentration (b). Species of crosslinking ion did have a significant effect on VEGF encapsulation efficiency (black bars, left y-axis) and total VEGF release (gray bars, right y-axis) (c). Like particle diameter, encapsulation efficiency was not affected by variation of Ca^{2+} (squares) and Zn^{2+} (triangles) crosslinker concentration. All data represents particles crosslinked by Cl^- salts at

700 mM unless otherwise noted, *** $P < 0.001$, ^data is statistically significant from all other comparable data sets ($P < 0.01$).

Fig. 3.

Alginate microparticle cytotoxicity to HUVEC *in vitro*. The effect of alginate microparticles on HUVEC viability is dependant on crosslinker ion species (a). Washing the burst of Zn²⁺ ions from 700 mM ZnCl₂-crosslinked particles significantly reduces particle cytotoxicity in a similar way to reducing the overall concentration of ZnCl₂ during particle formulation (b). The counter ion present in the Zn²⁺ salt used for crosslinking has an effect on particle cytotoxicity (c). #Difference in cytotoxicity profile for CaCl₂ was statistically significant compared to that of ZnCl₂ ($P < 0.05$) and BaCl₂ ($P < 0.01$), *Profiles for 700 mM ZnCl₂ post-wash and 90 mM ZnCl₂ were statistically different from 700 mM ZnCl₂ pre-wash ($P < 0.05$), as computed by a Bonferroni post-hoc test.

Fig. 4.

Choice of crosslinking ion species alters VEGF release from alginate microparticles. *In vitro* release profiles of VEGF from small alginate microparticles crosslinked by BaCl₂ (a), SrCl₂ (b), CaCl₂ (c), ZnCl₂ (d), Zn(O₂CCH₃)₂ (e), and ZnSO₄ (f) at the indicated concentrations.

Fig. 5.

Release mechanism of particles crosslinked by Zn^{2+} and Ca^{2+} . Release data for 700 mM CaCl_2 -crosslinked (a) and 700 mM (closed squares), 350 mM (circles), 175 mM (open squares), and 90 mM (diamonds) Zn^{2+} -crosslinked (b) particles were fit to the power law model for drug release, data summarized in (c) (values for n expressed as mean $\pm$ 95% confidence interval value).

Fig. 6.

Mixed populations of particles crosslinked by Zn^{2+} and Ca^{2+} . Mixing particles formed by crosslinking with 700 mM ZnCl_2 and 700 mM CaCl_2 at various ratios results in intermediate release profiles of VEGF (a). Combining Zn^{2+} and Ca^{2+} ions into the same crosslinking solution results lower overall VEGF release dominated by Zn^{2+} crosslinking (b). A 1:1 mixture of 700 mM ZnCl_2 - and 700 mM CaCl_2 -crosslinked particles displays a cytotoxicity profile similar to the of particles crosslinked by 700 mM CaCl_2 alone (c). Bioactivity of encapsulated VEGF is not significantly affected by choice of crosslinking ion species or combination of particles crosslinked by different species (d). For all formulations, VEGF bioactivity remains high relative to native VEGF for at least 2 weeks (positive control, top dashed line; bottom dashed line indicates negative control (no VEGF in media)).