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Controllable Soluble Protein Concentration Gradients in Hydrogel Networks**

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Abstract

Here we report controlled formation of sustained, soluble protein concentration gradients within hydrated polymer networks. The approach involves spatially localizing proteins or biodegradable, protein-loaded microspheres within hydrogels to form a protein-releasing “depot”. Soluble protein concentration gradients are then formed as the released protein diffuses away from the localized source. Control over key gradient parameters, including maximum concentration, gradient magnitude, slope, and time dynamics, is achieved by controlling the release of protein from the depot and subsequent transport through the hydrogel. Results demonstrate a direct relationship between the amount of protein released from the depot and the source concentration, gradient magnitude, and slope of the concentration gradient. In addition, an inverse relationship exists between the diffusion coefficient of protein within the hydrogel and the slope of the concentration gradient. The time dynamics of the concentration gradient profile can be directly correlated to protein release from the localized source, providing a mechanism for temporally controlling gradient characteristics. Therefore, each key, biologically relevant parameter associated with the protein concentration gradient can be controlled by defining protein release and diffusion. We anticipate that the resulting materials may be useful in three-dimensional cell culture systems, and in emerging tissue engineering approaches that aim to regenerate complex, functional tissues.

Keywords

molecular transport; biomaterials; tissue engineering; PEG; PLG

1. Introduction

The existence of protein concentration gradients and their effects has been observed in a variety of biological systems during tissue development and regeneration[1–3]. Spatial gradients in the concentrations of soluble proteins (e.g. growth factors) play a key role in cell survival[4–6], proliferation[6–8], and differentiation[7,9,10], and variations in their local concentrations ultimately dictate tissue structure and function. Pattern formation in the developing chick limb is an illustrative example in which the 48 kDa protein sonic hedgehog (shh) is secreted by a localized group of cells termed the “zone of polarizing activity” (ZPA) [11]. Diffusion of shh away from the ZPA results in establishment of a shh concentration gradient, which has been shown to elicit direct, long-range effects on the proliferation and differentiation of cells in presumptive limb tissue[12]. This general mechanism for gradient formation, in which a localized cell source secretes a protein that diffuses away and generates a resulting spatial concentration gradient, is common in nature, and has also been

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implicated in vertebrate neural tube axis specification[13,14], drosophila imaginal wing disc formation[15], and early xenopus embryo patterning[16].

The importance of protein gradients in natural systems suggests their potential for influencing the behavior of cells embedded within functional, synthetic biomaterials. Mimicry of these gradients could be particularly important in functional tissue engineering approaches, which aim to use combinations of cells, materials, and signaling molecules to recreate natural tissue structure and function. One recent approach used to generate protein gradients in materials involves exposing cells to covalently immobilized growth factors in specified locations within hydrogel networks[17–21]. This type of approach has been used to expose cells to spatial gradients of nerve growth factor[17], basic fibroblast growth factor[18], and epidermal growth factor[19]. Results indicate that covalently-immobilized protein gradients influence fibroblast alignment[20], vascular smooth muscle cell alignment and migration[18], axonal guidance[17] and neurite extension[21]. Taken together, these previous studies suggest a role for protein gradients in both wound healing and emerging tissue engineering applications. However, the types of molecules that can be effectively presented to cells using covalent immobilization may be limited, as protein function can be compromised upon covalent modification[22,23], and covalent immobilization can affect cellular uptake of proteins, an important step in many cell signaling pathways[24].

In an alternative set of studies, microfluidic systems based on laminar flow and mixing have been used to generate soluble protein gradients[25]. Chung and coworkers have used microfluidic gradient generators to show that soluble gradients in epidermal growth factor, fibroblast growth factor, and platelet-derived growth factor influence proliferation and differentiation of human neural stem cells in a spatially-defined manner[26]. These microfluidic approaches provide important insights into the effects of protein gradients on cell behavior, but are constrained to size limits required for laminar flow and thus are difficult to adapt for three-dimensional cell culture and tissue engineering applications. Previous approaches have not yet attempted to directly mimic the abovementioned natural mechanism for gradient establishment, in which a soluble protein is secreted from a localized source over an extended timeframe in a controlled manner, and the subsequent diffusion of the protein through the natural tissue establishes a spatial concentration gradient.

Since the concentration ranges in natural protein gradients can span orders of magnitude and can persist for days, the ability to control the magnitude, slope, and persistence time of a protein concentration gradient is crucial. These characteristics of soluble protein gradients have been difficult to control in synthetic systems[27]. Here we present an approach to form tunable soluble protein gradients, inspired by the aforementioned mechanism for protein gradient formation in some natural biological systems. The approach entails forming a spatially localized, protein-releasing region within a hydrogel network. More specifically, we have created poly(ethylene glycol) (PEG) hydrogels containing geometrically-defined regions of proteins or protein-releasing poly(lactide-co-glycolide) (PLG) microspheres. These regions, referred to here as “depots”, serve as a prolonged and tunable source of soluble protein. As protein diffuses away from the localized depot through the hydrogel network, a concentration gradient is formed. Here we report that controllable parameters dictating protein release from the depot and protein diffusion through the hydrogel strongly influence the resulting soluble protein gradient characteristics. The resulting materials are expected to serve as an adaptable platform system for three-dimensional cell culture and tissue engineering applications.

2. Results

2.1 Concentration Gradient Formation

Spatial localization of protein-releasing depots within a three-dimensional hydrogel network resulted in the formation of soluble protein concentration gradients. Bright field microscopy (Fig. 1) indicated that protein-releasing microspheres were localized within a depot and separated from the rest of the hydrogel, creating a planar boundary. When subjected to mechanical strain these materials did not preferentially fail at the depot boundary (data not shown), indicating that the hydrogel network was continuous. When these depots contained rhodamine-labeled bovine serum albumin (BSA) (excitation maximum: 544 nm, emission maximum: 576 nm) (Fig. 2a) or PLG microspheres loaded with rhodamine-labeled BSA (Fig. 2b), the resulting hydrogels contained a spatial gradient in fluorescence emission. Gradients in fluorescence emission displayed a repeatable shape, characterized by high fluorescence intensity closest to the depot with a sharp decrease leveling out over 1–3 mm before obtaining baseline levels. Networks containing PLG microspheres devoid of protein showed no measurable fluorescence (Fig. 2c), as expected. Importantly, hydrogels containing depots loaded with poly(styrene) microspheres modified with a covalently-linked and therefore non-diffusive fluorophore (*Peakflow* flow cytometry beads, Invitrogen, emission maximum: 575nm) displayed local fluorescence within the depot boundaries and minimal fluorescence outside of the depot (Fig. 2d). When fluorescent intensity was plotted as a function of distance from the protein-releasing depot (Fig. 2e), comparisons between conditions showed that the fluorescence measured in the non-diffusive fluorophore conditions was negligible. Taken together, these data indicate that the spatial gradients in fluorescence emission observed in samples containing protein-releasing depots can be attributed to a spatial gradient in [BSA].

To assist in the description of soluble protein gradient characteristics in the following paragraphs we define the following concentration gradient parameters: 1) Source protein concentration = [protein] within the hydrogel at the depot boundary; 2) Magnitude = the difference between [protein] within the depot boundary and [protein] 3 mm away from the depot boundary; 3) Average slope = the average rate of concentration decrease between the depot boundary and 3 mm away from the depot boundary. 4) Initial slope = the average rate of concentration decrease over the first 10%, or 0.3 mm, from the depot boundary; and 5) Normalized initial slope = the slope over the first 10% normalized to the value of the source concentration. All calculated slopes had negative values, thus only magnitudes are reported.

2.2 Influence of Depot Release Characteristics on Gradient Parameters

The concentration of protein-loaded microspheres present in the depot influenced the protein release profile, which in turn influenced the characteristics of soluble protein concentration gradients. Fluorescence micrographs obtained after 2 days (Figs. 3a–c) and quantification of BSA concentration as a function of distance from the depot (Fig. 3d) displayed a clear contrast in gradient profiles as the concentration of microspheres within the depot was increased. Ovalbumin gradient profiles were similarly dependent on the microsphere concentration in the depot, indicating that formation of gradients was not specific to BSA (Fig. 3e). Characteristic gradient parameters, including source concentration, magnitude, average slope, and initial slope, each increased significantly as the microsphere concentration in the depot was increased (Table 1). To gain insight into the relationship between protein release from the depot and gradient characteristics, BSA release into solution from isolated depots was also measured. As expected, the amount of protein released into solution from the depots also increased as the concentration of BSA-loaded microspheres within the depots was increased (Fig. 3f), indicating that protein release from the source strongly influences protein concentration gradient characteristics.

2.3 Influence of Protein Size and [PEGDA] on Gradient Characteristics

Protein size influences diffusion through the hydrogel network, thereby strongly influencing the soluble protein concentration gradient characteristics (Fig 4). Three model proteins, lysozyme (MW = 14 kDa, $R_h = 20 \text{ \AA}$ [28,29]), ovalbumin (MW = 44 kDa, $R_h = 29 \text{ \AA}$ [30,31]), and BSA (MW = 64 kDa, $R_h = 36 \text{ \AA}$ [32–34]) were chosen for analysis based on their distinct hydrodynamic radii. Experiments in which rhodamine-labeled proteins were released from thin, cylindrical PEG hydrogels (height = 1 mm, diameter = 5 mm) indicated that protein transport within the hydrogels was dependent on the size of the protein, with smaller proteins releasing more rapidly than larger ones (Fig 4a). In particular, these release studies resulted in calculated diffusion coefficients of $5.2 \times 10^{-8} \text{ cm}^2/\text{s}$, $2.8 \times 10^{-8} \text{ cm}^2/\text{s}$, and $1.3 \times 10^{-8} \text{ cm}^2/\text{s}$ for lysozyme, ovalbumin, and BSA, respectively. Protein gradient characteristics were similarly dependent on protein size, as the concentration gradient slope was steeper for BSA than for lysozyme, with ovalbumin lying between the two extremes (Fig 4b). This result was quantified (Table 2) by examining the normalized initial slopes of the concentration gradients for the three model proteins (BSA = 1.4 mm^{-1} , ovalbumin = 1.2 mm^{-1} , and lysozyme = 1.0 mm^{-1} , respectively). Interestingly, there was an inverse relationship between protein diffusion coefficient within the hydrogel and the normalized initial slope of protein concentration gradients resulting from the three model proteins (Fig. 4c), indicating that protein size is a key determinant of soluble gradient characteristics.

[BSA] gradient characteristics were also dependent on the PEGDA concentration in the hydrogel precursor solution (Fig 4d). This result was quantified by examining the normalized initial slopes of [BSA] gradients in PEG hydrogels formed with 5% PEGDA (normalized initial slope = 1.4 mm^{-1}), 10% PEGDA (1.9 mm^{-1}), and 15% PEGDA (2.1 mm^{-1}) in the hydrogel precursor solution. This result further supports the assertion that protein diffusivity influences protein concentration gradients, as the mesh size in PEG hydrogels decreases with increasing PEGDA mass fraction in the precursor solution.

2.4 Time Dynamics of Concentration Gradients

Localization of soluble rhodamine-BSA depots in PEG hydrogels resulted in concentration gradient profiles that decreased significantly within 7 days (Fig. 5a). This trend was quantified by examining the time dynamics of the normalized source concentration (Fig. 5b), which monotonically decreased to less than 60% of its original value within 7 days. In contrast, localization of depots containing rhodamine-BSA-loaded PLG microspheres resulted in a significant increase in gradient persistence time. Importantly, the time dynamics of soluble (BSA) concentration gradients are directly related to the kinetics of protein release from the localized depot. This correlation is particularly apparent when comparing fluctuations in concentration gradient profiles within a hydrogel containing 30 mg/ml microsphere depots (Fig. 5c) to fluctuations in the release of BSA into solution from gradient hydrogels containing 30 mg/ml microspheres within the depot region (Fig. 5d). A low level of protein was released into solution from gradient hydrogels during day one ($9 \pm 1.2 \text{ pmol}$), which results in a shallow initial concentration gradient profile (◆ in Fig 5c). The amount of protein released increased significantly during day 2 ($31 \pm 1.7 \text{ pmol}$), which correlated with a steeper concentration gradient profile (■ in Fig. 5c). Finally, protein release underwent a gradual decrease from day 2 to day 6 ($11 \pm 1.3 \text{ pmol}$), which correlated with a gradual shallowing of the concentration gradient profile from day 2 to day 6 (Δ in Fig. 5c). This same trend – an increase from day 1 to day 2 and subsequent gradual decline – was evident in quantitative analysis of the gradient magnitude (Fig. 5e), source concentration (Fig. 5f), and initial slope (Fig. 5g). Taken together, these data suggest that the release profile from the source region, which is tunable, is a key determinant of soluble protein gradient characteristics in hydrogel networks.

3. Discussion and Conclusions

Soluble protein concentration gradients were formed via spatial localization of a sustained release depot within a hydrogel network. Specific characteristics of the gradients formed were tunable, and could be controlled by varying the amount of protein released from the depot or by modulating the molecular transport of protein through the material. The time dynamics of the concentration gradient profile were directly correlated to release of protein from depots, as fluctuations in protein release were mirrored by fluctuations in gradient characteristics (e.g. source concentration, concentration range, initial slope).

In order to quantify characteristics of the soluble protein concentration gradients formed, parameters such as source concentration, concentration range, average slope, and initial slope were defined and varied over a wide range. These variables were chosen because they are important during natural biological processes, and therefore likely to be important in potential biological applications of soluble protein gradients. For example, the affects of nerve growth factor gradients on axon guidance in vitro are dependent on both absolute concentration and the slope of the concentration gradient[17]; similar dependences have been observed in vivo when examining the affects of bone morphogenetic protein-4 and bicoid on xenopus ectoderm[35] and drosophila patterning[36], respectively. Based on these previous studies it is clear that cell responses to soluble protein gradients are dependent on both the absolute concentration and the rate of change of concentration within a local environment. The materials generated in the current studies are capable of controlling these parameters, which suggests that they may be useful as model systems to explore the influence of proteins (e.g. growth factors) on cell behavior and as matrices to support developing tissues.

The time dynamics of the gradients formed are directly related to protein release from the localized protein depot. The incorporation of protein-releasing microspheres as a source of protein results in sustained high levels of protein concentration (Fig. 5c, f) when compared to gradients formed using solely soluble protein in the depot (Fig. 5a, b). In addition, shifts in protein release from the source do not merely result in translational shifts in the concentration gradient profile but instead result in changes in other quantifiable gradient characteristics, such as the source concentration, magnitude, and the slope of the concentration gradients. The ability to tune the properties of a concentration gradient over time by varying release kinetics provides an adaptable mechanism for temporal control. It is noteworthy that a wide range of biodegradable polymers, including poly(anhydrides), poly(α -hydroxy acids), and materials composed of their copolymers, have been used in previous studies to release proteins over controllable time intervals ranging from days[37] to weeks[38]. Therefore, by incorporating this broad range of biodegradable polymers into the gradient hydrogel system described here it may ultimately be possible to vary the time dynamics of protein concentration gradients over a wider range. The ability to tune the time dynamics of gradients may be important in biological applications, as gradients illicit their effects over a variety of timeframes in natural systems. For example, protein gradients that are believed to determine dorsal-ventral patterning in neural tube formation[39] and limb patterning in the chick embryo[11] produce their effects over the span of up to one week, whereas gradients that dictate analogous developmental events in humans span days to weeks[40]. Furthermore, the effects of protein gradients on bone formation via endochondral ossification[41] span weeks to months.

The approach developed in this study differs from previous approaches that have used covalent protein immobilization to create concentration gradients within synthetic materials[17–21]. Covalent immobilization studies demonstrate a high level of control over initial protein concentration gradients, but the time dynamics of the gradients are not

inherently controllable. In contrast, the approach described herein releases soluble proteins, and the characteristics of resultant protein gradients are therefore dictated by system geometry, protein release from the source, and protein diffusion through the network. The adaptability of each of these parameters suggests that the current approach can be tailored and, importantly, the general approach presented here is likely applicable to a broad range of proteins and hydrogel network chemistries.

The results of this study are consistent with previous studies of protein transport through PEG-based hydrogel networks, which have implicated Fickian diffusion as the dominant mode of protein transport[42–45]. Two parameters primarily determine the numerical solution of the Fickian diffusion relation – the protein diffusion coefficient within the hydrogel network and the boundary conditions. This study demonstrates that variation of the parameters that influence protein transport within the hydrogel, specifically protein size and network mesh size, results in control over the protein diffusion coefficient and the resulting protein gradient. Protein size was varied using proteins with differing hydrodynamic radii (BSA, ovalbumin, and lysozyme), and there was a decrease in [protein] gradient slope with decreasing protein size (Fig. 4c). It is noteworthy that the isoelectric point of each protein studied here are different, so it is possible that protein-material interactions may have also influenced the gradient characteristics. There was also a decrease in [protein] gradient slope with decreasing weight percentage of PEGDA in the hydrogel precursor solution (Fig. 4d). This effect can be attributed to a decrease in protein transport within PEG hydrogels as the PEGDA weight percentage is increased, as the network mesh size decreases with increased PEGDA weight percentage [46–48]. Therefore, a similar phenomenon is observed when either the protein hydrodynamic radius or the hydrogel mesh size are varied – a decrease in the size of the protein relative to the mesh size of the hydrogel results in a more shallow concentration gradient. Other parameters, including the baseline [protein] in the surrounding solution, potentially provide additional mechanisms for controlling [protein] gradient characteristics, though they are not explicitly explored here. In addition, due to the relative ease of spatial patterning of protein-releasing microspheres within a hydrogel, it may be possible to create gradients within hydrogels of varying geometries and sizes (microns to centimeters). Therefore, this approach could be useful in applications such as functional tissue engineering that require widely variable spatial dimensions, where other, laminar flow-dependent systems (e.g. microfluidic environments[25,26]) are dimensionally limited.

4. Experimental

4.1 Microsphere Synthesis

Proteins were encapsulated within poly(lactide-co-glycolide) (PLG) microspheres using a modified version of a well-characterized water-in-oil-in-water (w/o/w) double emulsion/solvent evaporation technique described elsewhere[49]. Briefly, the primary emulsion was formed by sonicating a mixture containing the desired protein in PBS along with 5% poly (DL-lactide-co-glycolide) (PLG) (50:50, inherent viscosity = 0.15–0.25, Aldrich) in ethyl acetate. The secondary emulsion was created by adding the mixture described above to a vigorously stirring solution containing 1% poly (vinyl alcohol) (PVA) (MW = 9–10kDa, 80% hydrolyzed, Sigma-Aldrich) and 7% ethyl acetate in DI water. Following hardening via solvent evaporation, the microspheres were collected via filtration and washed with DI water.

4.2 Hydrogel Preparation

PEG chains were derivatized on each end with acrylate groups using methods described previously[50]. Briefly, a PEG (MW = 8 kDa, Fisher Scientific) solution in benzene was azeotropically distilled and reacted with a 4X molar excess of acryloyl chloride (Alfa

Aeasar) in the presence of 4X excess of triethylamine base. The resultant PEG-diacrylate (PEGDA) solution was filtered, precipitated via addition of cold diethyl ether and purified via dialysis against DI water.

Spatial patterning of microspheres within PEGDA hydrogels was accomplished using the following procedure, unless otherwise indicated. Hydrogel precursor solutions were composed of 15% (w/v) PEGDA, 0.05% photoinitiator (I2959, Ciba, Switzerland), and 0.5% Tween 20 in PBS. Polymer microspheres containing rhodamine-labeled proteins were suspended in PEGDA hydrogel precursor solutions at various concentrations (10–60 mg microspheres/ml precursor solution). 25 μ l of these solutions were added to 3mm $\times$ 3mm $\times$ 1mm rectangular polystyrene molds and exposed to UV light (4.5 mW/cm²) for 10 minutes. The resultant protein-releasing “depots” were rinsed in PBS and added to 50 μ l of hydrogel precursor solution in a separate 3mm $\times$ 3mm $\times$ 1mm polystyrene mold. Depots were allowed to float in precursor solution for 5 minutes, then exposed to UV light for 10 minutes. The resulting PEG hydrogels, or “gradient hydrogels” contained localized depots loaded with protein-releasing polymer microspheres.

4.3 Protein Release Studies

Protein release from the depot-containing hydrogels was carried out in order to gain insight into the dynamics of soluble protein concentration gradient formation. Protein release from the depot-containing hydrogels during incubation in PBS was characterized by measuring emission from fluorescently-tagged proteins in the solution. Hydrogels were incubated in 1 ml of PBS, refreshed daily, within an aluminum foil-covered container constantly agitated at room temperature. Protein concentrations in solution were calculated via comparison to linear standard curves relating fluorescence emission to fluorescently-tagged protein concentration ($r^2 > 0.98$, see supplementary information S.I. 1)

To determine protein diffusion coefficients in hydrogel networks, protein release from thin cylindrical (thickness = 1 mm, diameter = 8mm) hydrogels was measured via fluorescence emission and diffusion coefficients were calculated using the following relation[51]:

$$\frac{M_t}{M_\infty} = 2 \left[\frac{D_e t}{\pi \delta^2} \right]^{1/2} \quad (2)$$

where M_t/M_∞ is the fractional mass release, D_e is the diffusion coefficient, and δ is the thickness of the cylindrical hydrogel.

4.4 Characterization of Protein Gradients

All model proteins were covalently tagged with NHS-Rhodamine (Pierce) for detection via fluorescence microscopy. Hydrogels containing protein-loaded depots were kept, individually, in 1 ml PBS. Fluorescence images of the gradient gels were captured using a Hamamatsu IEEE 1394 Digital CCD Camera connected to an Olympus IX51 inverted compound microscope. Fluorescence intensity was measured as image grey level, and converted to protein concentration within the gel via comparison to a linear standard curve relating grey level to fluorescently tagged protein concentration in a hydrogel ($R^2 > 0.997$, see supplementary information S.I. 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Spatial localization of PLG microspheres within hydrogel network. A) Schematic representation and B) bright field image of PEGDA hydrogel containing distinct “depots” loaded with PLG microspheres (left side of hydrogel). Scale bar = 1mm

Figure 2.

Soluble protein concentration gradients in a hydrogel network. The fluorescence micrographs are pseudocolor images of hydrogels containing depots loaded with A) Rhodamine-BSA, B) PLG microspheres releasing rhodamine-BSA, C) PLG microspheres containing no fluorophore, or D) poly(styrene) beads containing a non-diffusive fluorophore. Depots are located directly to the left of the images and are not shown. Images were taken two days after hydrogel preparation. Scale bar = 1mm. E) Comparison of fluorescence intensity profiles for A ($\diamond$), B ($\blacklozenge$), C ($\blacktriangle$), and D ($\blacksquare$).

Figure 3.

The effect of microsphere mass within protein depots on concentration gradient characteristics. Pseudocolored fluorescence micrographs displaying observed gradients within hydrogels containing depots with A) 10 mg/ml, B) 30 mg/ml, and C) 60 mg/ml BSA loading. Scale bar = 1mm. The effect of microsphere mass within the depot on D) [BSA] gradient profile and E) [Ovalbumin] gradient profile. F) Release of BSA into solution from gradient hydrogels. 60 mg/ml (◆), 30 mg/ml (■), 10 mg/ml (▲).

Figure 4.

Effect of protein size and network mesh size on concentration gradient profile. A) Fractional release from thin PEG hydrogel cylinders for lysozyme (◆), ovalbumin (■), and BSA (▲). B) Day 1 normalized concentration gradients for lysozyme (◆), ovalbumin (■), and BSA (▲). C) Relationship between protein diffusion coefficient, calculated from protein release experiments in (A) and normalized initial slope of protein gradients. D) Day 1 normalized [BSA] gradients in PEG hydrogels prepared with 5% PEGDA (◆), 10% PEGDA (■), and 15% PEGDA (▲).

Figure 5.

Time dynamics of concentration gradients. Fluctuations in A) concentration gradient profile and B) normalized source concentration over time in gradient hydrogels with depots containing soluble rhodamine-BSA. C) Fluctuations in concentration gradient profile in gradient hydrogels with depots containing rhodamine-BSA-releasing PLG microspheres. D) Daily release from gradient hydrogels, *g*, and depots not included into gradient hydrogels, *d*. Variations in E) magnitude, F) source concentration, and G) initial slope in gradient hydrogels containing 60 (◆), 30 (■), and 10 (▲) mg/ml microspheres within the depots.

Table 1

Soluble [BSA] gradient parameters are dependent on the amount of microspheres incorporated within the protein depot. See results section for parameter definitions

Microsphere concentration (mg/ml)	60	30	10
Concentration at source (nM)	550 ± 11	319 ± 8	107 ± 3
Magnitude (max-min) (nM)	500 ± 40	290 ± 30	90 ± 15
Initial Slope (0–10%) (nM/mm)	700 ± 15	460 ± 30	150 ± 11
Average Slope (nM/mm)	170 ± 15	100 ± 11	29 ± 5

Table 2

Transport within hydrogels and, in turn, soluble protein concentration gradient parameters, are dependent on protein size.

Quantity	Lysozyme	Ovalbumin	BSA
Molecular weight (kDa)	14	44	64
Hydrodynamic radius (Å)	21[28,29]	28[30,31]	36[32–34]
Diffusion Coefficient (cm/s)×10 ⁸	5.2	2.8	1.4
Normalized initial slope (mm ⁻¹)	0.98 ± 0.10	1.19 ± 0.08	1.41 ± 0.03