Published in final edited form as:

J Control Release. 2016 May 28; 230: 1–12. doi:10.1016/j.jconrel.2016.03.032.

Local retention of antibodies *in vivo* with an injectable film embedded with a fluorogen-activating protein

Wen Liu^a, Matthew J. Saunders^{c,d,1}, Christina Bagia^a, Eric C. Freeman^e, Yong Fan^{f,d}, Ellen S. Gawalt^{b,g}, Alan S. Waggoner^{c,d}, and Wilson S. Meng^{a,*}

^aDivision of Pharmaceutical Sciences, Duquesne University, Pittsburgh, PA 15282, United States

^bDepartment of Chemistry and Biochemistry, Duquesne University, Pittsburgh, PA 15282, United States

^cMolecular Biosensor and Imaging Center and Carnegie Mellon University, Pittsburgh, PA 15213, United States

^dDepartment of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA 15213, United States

^eCollege of Engineering, University of Georgia, Athens, GA 30602, United States

^fInstitute of Cellular Therapeutics, Allegheny-Singer Research Institute, Pittsburgh, PA 15212, United States

^gMcGowan Institute for Regenerative Medicine, Pittsburgh, PA 15213, United States

Abstract

Herein we report an injectable film by which antibodies can be localized *in vivo*. The system builds upon a bifunctional polypeptide consisting of a fluorogen-activating protein (FAP) and a β -fibrillizing peptide (β FP). The FAP domain generates fluorescence that reflects IgG binding sites conferred by Protein A/G (pAG) conjugated with the fluorogen malachite green (MG). A film is generated by mixing these proteins with molar excess of EAK16-II, a β FP that forms β -sheet fibrils at high salt concentrations. The IgG-binding, fluorogenic film can be injected *in vivo* through conventional needled syringes. Confocal microscopic images and dose–response titration experiments showed that loading of IgG into the film was mediated by pAG^{MG} bound to the FAP. Release of IgG *in vitro* was significantly delayed by the bioaffinity mechanism; 26% of the IgG were released from films embedded with pAG^{MG} after five days, compared to close to 90% in films without pAG^{MG}. Computational simulations indicated that the release rate of IgG is governed by positive cooperativity due to pAG^{MG}. When injected into the subcutaneous space of mouse footpads, film-embedded IgG were retained locally, with distribution through the lymphatics impeded. The ability to track IgG binding sites and distribution simultaneously will aid the optimization of local antibody delivery systems.

*Corresponding author at: Duquesne University, 600 Forbes Avenue, 413 Mellon Hall, Pittsburgh, PA 15282, United States. Present address: Glycosensors & Diagnostics, JLABS, 3210 Merryfield Row, San Diego, CA 92121, United States.

Appendix A. Supplementary data: Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2016.03.032>.

Keywords

Bioaffinity film; Fluorescent imaging; Antibody formulation; Single-chain variable fragment; Mathematical simulation; Self-assembling peptide; Fluorogen-activating protein; EAK16; Dimerized light chain-5 (dL5); Controlled release; Draining lymph nodes

1. Introduction

Monoclonal antibodies are an expanding class of biotherapeutics [1]. While antibodies are typically formulated for intravenous or intramuscular administration, localized delivery can concentrate the biotherapeutics in diseased tissues, thereby increasing bioavailability to an extent that cannot be achieved through systemic routes [2]. Subcutaneous or intramuscular delivery can target regional lymph nodes in order to modulate local immune milieu [3]. Outcomes of inflammation are governed in part by the phenotypes of granulocytes, myeloid cells, and lymphocytes trafficking between injured tissues and draining lymph nodes. *In situ*-forming hydrogels are used to create depots of antibody drugs *in vivo*. Local administration of immune modulators can enhance efficacy and reduce adverse events. In-situ forming hydrogels or coacervates formed by cross-linked polymer chains that remain physically intact with high water intake are particularly attractive because of their ability to adapt to tissue space [2]. Drug loading can be accomplished by introducing the biologic during polymer cross-linking. The pore sizes (5–100 nm) and the resulting high permeability of such depots [4], however, preclude the ability to concentrate antibodies at injection sites and accumulating in draining lymph nodes. Rapid diffusion of drugs out of the network (“burst release”) can be limited by increasing cross-linking density [5] or through bioaffinity retardation [6]. A less explored challenge is that the location of the depot cannot be ascertained *in vivo*, making it difficult to determine the rate-limiting mechanism of drug localization.

The EAK16 series of β -sheet fibrillizing peptides (β FP) are used to formulate depots of small molecule drugs, proteins, and cells [7–9]. The β FP EAK16-II, comprising the sequence AEAEAKAKAEAEAKAK (with A as alanine, E as glutamic acid, and K as lysine), self-assembles into cross-linking fibrils in high ionic strength aqueous environment [7]. When administered through conventional needled syringes, EAK16-II rapidly forms stable coacervates *in vivo*, adapting and adhering to the local tissue contours [10]. Due to the high water content (~99.5% w/v) [7], IgG can be loaded into the fibrillar matrices with high efficiency. The amphiphilic EAK16-II, carrying no net charge at pH 7.4, presumably interacts with IgG *via* non-specific interactions, resulting in a large fraction of the protein molecules remaining unbound [11]. In hydrogels made with the β FP sequence RADA, drug release kinetics is governed by the molecular size of the encapsulated protein; IgG, the largest molecule tested, reaches maximum release by 24 h [12]. Koutsopoulos et al. subsequently demonstrated that burst release can be suppressed by encapsulating IgG in double-layer nanofibers using two amphiphilic building blocks, RADA and KLDL [13]. These studies demonstrate the versatility of β FP in controlling the release of protein drugs.

Biomolecules provide affinity mechanisms for tuning protein release from hydrogel matrices. The Sakiyama–Elbert group has engineered heparin-embedded hydrogels as

protein delivery systems. As highly sulfated glycosaminoglycans, heparins bind growth factors through ionic reversible interactions. These systems mimic reservoirs of endogenous growth factors in extracellular matrix [14–19] in which secreted proteins are protected from denaturation and aggregation. Hydrogels functionalized with aptamers have been used to control protein release [20–23]. Diverse sequences of aptamers can be generated to confer different ligand specificities and affinities. The Shoichet group has used Src homology domain 3 (SH3) to tune the release of growth factors fused with SH3 ligands [24–27]. In these designs, rapid diffusion is suppressed; instead, drug release is a function of the affinity of the binding interactions (K_D), the relative ratio of binding sites (ligands) to proteins, and the dissociation rate constant (k_{off}) of the protein-binding site complexes [26]. Thus affinity-based hydrogels afford the ability to tune release kinetics by adjusting classical biochemical parameters.

We have used the coassembly method to engineer biochemical functionalities into EAK16-II fibrillar matrices [10,28–31]. Using the analog EAKIIH6, His-tags are incorporated into EAK16-II fibrils, driven by the β FP domain for β -sheets. The two peptides co-assemble efficiently, with greater than 90% of EAKIIH6 incorporated into the composite [10] generated from excess EAK16-II. By embedding recombinant protein A/G (pAG) *via* anti-His-tag antibodies, IgG can be loaded and release over time [29–31]. Because Protein A and Protein G bind to IgG with relative high affinities (with apparent binding constants reported at 2.9×10^7 and $1.13 \times 10^8 \text{ M}^{-1}$ [32], respectively), pAG provides a stable affinity mechanism, allowing diffusive release of the IgG *in vivo* driven by the concentration gradient between the depot and the open system of surrounding tissues and lymphatics. *Staphylococcal* Protein A (SpA) has been developed into a systemic therapeutic agent (PRTX-100) recently approved for use in humans [33]. The system renders rapid formation of depots and retention of IgG molecules in subcutaneous injection sites for up to ten days [29]. The EAK16-II/EAKIIH6 system, when adapted with pAG and anti-His-tag antibodies, was designed as antibody-functionalized scaffolds for cytokines and cells [29,30]. The ability to monitor IgG-binding sites *in vivo* will aid efforts in correlating *in vitro* and *in vivo* observations.

Herein we described an *in situ*-forming film on which the binding sites for IgG can be monitored using an activation-dependent fluorescent module. The films are generated by intermixing EAK16-II with its analog dL5_EAK, a polypeptide consisting of a FAP with two light chains, called dL5, fused to three repeats of AEAEAKAK *via* a glycine–serine spacer, (GGGS)₃ [28]. In high ionic strength environment, EAK16-II and dL5_EAK undergo solution–film transition and coassembly, apparently simultaneously. The resultant materials are illuminated in the presence of malachite green (MG), the fluorogen activated by dL5 [34]. Relative to free MG, the fluorescence of dL5-bound MG is enhanced by up to 20,000 folds, due to constraining of the rotatable bonds in MG by two L5 proteins. Crystallographic studies have shown that MG mediates dimerization of two L5 proteins to form a fluorescent ternary complex [35]. We hypothesized that the system of MG-conjugated pAG (pAG^{MG}), dL5_EAK and EAK16-II, when deployed together (called dL5 film), serves as a self-illuminating, injectable formulation for local deposition of IgG. The tight binding of dL5 to MG ensures a stable intermediate [35]. Release of IgG from pAG^{MG} is driven by the diffusion of free IgG into tissues surrounding the injection site. The

exceptionally low fluorescence background renders the fluorogenic module an efficient reporter of binding sites, independent of model IgG drugs [34]. We characterized and modeled the release kinetics *in vitro*, and found that the dL5 film retained IgG at the injection site and draining lymph node *in vivo*.

2. Materials and methods

2.1. EAK16-II and expression of dL5_EAK

EAK16-II was custom synthesized by American Peptide Company (Sunnyvale, CA) at greater than 95% purity with the N-terminus acetylated and C-terminus amidated. The sequence was confirmed by mass spectrometry by the manufacturer. The peptide, received lyophilized, was reconstituted in sterile deionized water (18.2 MΩ at 25 °C) as needed and stored at −80 °C until use. Construction of plasmids encoding dL5_EAK was described previously [28]. Expression and purification of dL5_EAK was carried out by transforming pET21 GST-HRV-dL5_EAK into calcium competent Rosetta-Gami 2(DE3) *Escherichia coli*. (Novagen, Madison, WI). The transformed bacteria were grown in 500 mL volumes and induced using 500 μM IPTG and 0.4% glucose overnight at 20 °C. Proteins in cell lysates were captured using Ni-NTA agarose columns (Qiagen, Valencia, CA) and eluted by HRV3C protease. Fractions containing the protein were combined and dialyzed against PBS, with the resultant concentrate quantified using UV-Vis ($\lambda = 280$ nm). Details of plasmid construction, protein expression and purification, can be found in Saunders et al. [28].

2.2. Conjugation of Protein A/G

Protein A/G (pAG) and malachite green isothiocyanate (MG-ITC) were purchased from Life Technologies (Carlsbad, CA). Conjugation of pAG to MG-ITC was carried out in alkaline aqueous buffers (pH = 8.5 to 9.5), with the resultant conjugate separated from unreacted MG-ITC using PD-10 columns (Sephadex G-25M, GE Healthcare, Aurora, OH). The degree of labeling of the eluent was calculated based on the molar ratio of dye concentration to protein concentration. Each mole of pAG was conjugated with 0.67 mol of MG, as determined by bicinchoninic acid assay for the former and UV-Vis absorbance at 633 nm of the latter (Supplemental material S1).

2.3. Gel electrophoresis

Different amounts of EAK16-II (10 μL, 20 μL, 40 μL, 80 μL or 160 μL, at 5 mg/mL) were added to 8.3 μL of dL5_EAK (72 μM) to prepare mixtures containing EAK16-II to dL5_EAK at 50:1, 100:1, 200:1, 400:1, and 800:1 M ratios. Each sample was suspended in a total volume of 500 μL, followed by incubation at 37 °C overnight. The samples were centrifuged at 16,000 RFC for 6 min with the supernatants analyzed using denaturing Novex NuPAGE 4–12% Bis-Tris gels. To each sample, the supernatant (5 μL) was added with 5 μL of NuPAGE SDS buffer and 10 μL of PBS, with the resulting solution heated at 70 °C for 10 min before loading. All samples were run at 200 V for 35 min in NuPAGE MES SDS running buffer. Proteins were visualized using the SilverQuest™ staining kit for which a detection limit of 0.3 ng has been reported. All electrophoretic reagents were purchased from Life Technologies (Carlsbad, CA). Images were captured using the Kodak 440 Image Station (Kodak, Rochester, NY), and band intensities were quantified using the NIH Image J

software. Percent dL5_EAK incorporated into the insoluble fraction was quantified by subtracting the intensity of the protein band in samples containing EAK16-II from the intensity of the samples without EAK16-II.

2.4. Viscosity measurement

Stock solutions of EAK16-II and dL5_EAK were prepared in distilled water at the level of 10 mg/mL and 12.2 μ M respectively. Samples of EAK16-II with or without dL5_EAK were prepared in PBS (pH = 7.4) and drew into positive displacement pipettes (RheoSense Inc., San Ramon, CA). EAK16-II was prepared in different concentrations (0.01–2 mg/mL) in two solvents (PBS and distilled water). The EAK16-II with dL5_EAK in PBS was prepared at the ratio 400:1 and the final concentrations were 1.5 mg/mL and 2.3 μ M, respectively. Measurements were performed using the μ VISC unit (RheoSense Inc.) equipped with a temperature control module, which was set at 25 °C. Samples were subjected to different shear rates by changing the flow rate through the capillaries (depth = 50 μ m) in the sensor cartridge (HA01-01). The instrument is capable of measuring viscosity between 0.2 to 100 mPa·s from shear rates 6.5 to 5850 s⁻¹ at within 2% accuracy according to the manufacturer.

2.5. Titration of pAG^{MG} to dL5_EAK film

Films composed of EAK16-II and dL5_EAK (400:1) were established in Corning FluoroBlok 96-well plate (Thermo Scientific, Inc.). Different amounts of pAG^{MG} were added to the films to generate samples containing dL5_EAK to pAG^{MG} molar ratios of 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1, or 16:1. Sufficient PBS was added to each well to attain a final total volume of 200 μ L. The solvated films were incubated overnight at 37 °C before measuring MG/dL5 fluorescence using a Perkin Elmer 1420 microplate reader, with excitation filter at 635 nm and emission filter at 670 nm. EAK16-II with pAG^{MG}, or EAK with a PEGylated form of MG [34] were used as the controls (n = 3).

2.6. Laser-scanning confocal microscopy

Samples were prepared for confocal imaging as described below. To 20 μ L of 5 mg/mL EAK16-II, 40 μ L of 10 μ M dL5_EAK were added and mixed to produce a gel with a molar ratio of 150:1. To this mixture, 12.2 μ L of 11 μ M pAG^{MG}, 4 μ L of 0.05 mg/mL FITC-labeled rat anti-mouse CD23 IgG anti-body were added in a confocal dish (MatTek Corp., Ashland, MA). The sample was brought up to 100 μ L with PBS and incubated overnight. The film was collected by centrifugation and washed with 200 μ L of fresh PBS five times before transferring to a glass bottom microwell dish. Imaging was performed using a Zeiss Axioplan 2 imaging microscope equipped with a mercury lamp. Fluorescence was collected a 470/530 nm filter, and Cy5 fluorescence was obtained using a 710/75 nm filter (Chroma, Lititz, PA). Control samples without dL5_EAK, without pAG^{MG}, or containing only EAK16-II and fluorescein-labeled IgG, were also imaged.

2.7. In vitro IgG release

The kinetics of release of a fluorescein-labeled antibody (Pierce goat anti-human IgG) from pAG^{MG}-embedded dL5 films were determined by monitoring fluorescence over time. The IgG-loaded materials were established in MicroSert (inner diameter = 4.15 mm, maximum

volume = 500 μL) by instilling a mixture of EAK16-II (60 nmol), dL5_EAK (150 pmol), pAG^{MG} (150 pmol), and IgG^{FITC} (75 pmol). The 50 μL mixture was incubated overnight at 37 °C before adding 400 μL of PBS (0.1% BSA, 0.025% NaN₃) as the release medium. The insert was placed in its receptacle vial and capped before incubation at 37 °C at the start of the release. At various time points, 200 μL of the release medium were collected for measurement (excitation = 485 nm; emission = 535 nm) using the Tecan Infinite M1000. Aliquots of fresh release medium were added to restore the system to its initial volume.

2.8. Modeling methodology

To delineate the parameters driving IgG release, a mathematical model is described and implemented here. The data for this comparison is taken directly from the *in vitro* case described in Fig. 4, in which diffusion of the antibody IgG from a tailored film with and without pAG^{MG} binding sites is compared. Six components in the system with four possible interactions were considered:

To understand the affinity interactions on the overall release dynamics, we considered the systems of equations previously described for other affinity systems designed for sustained release of proteins and drugs [6,15,25,36]. These models contain parameters analogous to those describe the current system in which two sets of reversible interactions operate: binding of pAG^{MG} to dL5_EAK, and binding of IgG to pAG^{MG}. Free pAG^{MG} would bind IgG, generating IgG-pAG^{MG} complexes that bind to dL5 associated with EAK16-II fibrils. All dL5_EAK are assumed remaining in the main EAK16-II fibrils throughout. The relative fractions of these components are governed by the rate constants associated with identifiable processes: with $k_1 > k_2$, and $k_7 > k_8$. Because pAG^{MG} was found to be relatively immobile compared to the IgG (Fig. 3b), the concentration of pAG^{MG} in the sample was held constant. This allows for the isolated study of IgG diffusion for describing the behavior of the system, focusing on the third reaction in Eq. set (1).

The diffusion of unbound IgG from the films is first modeled through Fick's law in a heterogeneous medium as shown in Eq. (2), where $[IgG]$ is the molar concentration of unbound IgG, and D is the variable diffusion coefficient. Diffusion is assumed to be one-dimensional, and the film and aqueous regions are assumed to be divided neatly into two cylindrical volumes as depicted in Fig. 4a.

$$\frac{\partial [\text{IgG}]}{\partial t} \Big|_{\text{diff}} = D \frac{\partial^2 [\text{IgG}]}{\partial x^2} \quad (2)$$

The diffusion equation is solved using central finite-difference methods. The film and release medium are discretized with respect to x with even grid spacing (100 points evenly distributed across the length of the vial). The change in IgG concentration at each node is then estimated and integrated through finite difference approximations (Eq. (3)) [37]. Zero-flux boundary conditions are enforced at either end of the vial. Since the diffusion coefficient is assumed to vary with location, the averaged value between the neighboring nodes is used.

$$\frac{\partial^2 [\text{IgG}]}{\partial x^2} = \frac{D_{i+1/2}([\text{IgG}]_{i+1} - [\text{IgG}]_i) - D_{i-1/2}([\text{IgG}]_i - [\text{IgG}]_{i-1})}{\Delta x^2} \\ [\text{IgG}]_i^{t+1} - [\text{IgG}]_i^t = \Delta t \frac{\partial^2 [\text{IgG}]}{\partial x^2} \quad (3)$$

The binding-release behavior of IgG from the pAG^{MG} sites is assumed to follow Hill's law [38], commonly used for ligand binding with multiple sites. This equation was selected due to uncertainty of the exact nature of the binding sites, by combining them into a single association constant with a variable describing their interactions. Each pAG^{MG} possesses six binding sites for IgG (4 on protein A, 2 on protein G), which may exhibit cooperative or competitive effects between the sites as binding progresses. Here n is Hill's coefficient, where $n > 1$ corresponds to positive cooperativity and $n < 1$ corresponds to negative cooperativity. $\bar{Y}$ is the ratio of bound sites to total sites, and K is the association constant.

$$\bar{Y} = \frac{K[\text{IgG}]^n}{1 + K[\text{IgG}]^n} \quad (4)$$

As the unbound IgG diffuses away from the hydrogel and into the surrounding medium, the equilibrium value for the bound pAG^{MG} complexed with IgG shifts as well, releasing a portion of the IgG to replace some of the diffused components. Therefore the total change in IgG concentration may be modeled as a combination of diffusion and release. The diffusion component is captured through Eqs. (2 and 3), and the release component may be calculated by taking the derivative of Eq. (4) with respect to the concentration of unbound IgG and multiplying by the total change in concentration [39,40]. This produces the final system of equations summarized as Eq. (5). It is assumed that IgG does not specifically interact with the dL5 dendrimers; instead the hydrogel structure provides a lower diffusion coefficient D within the hydrogel region of the model taken from Table 2.

$$\begin{aligned}
\frac{\partial[\text{IgG}]}{\partial t}_{\text{total}} &= \frac{\partial[\text{IgG}]}{\partial t}_{\text{diff}} + \frac{\partial[\text{IgG}]}{\partial t}_{\text{release}} \\
\frac{\partial[\text{IgG}]}{\partial t}_{\text{diff}} &= D \frac{\partial^2[\text{IgG}]}{\partial x^2} + \frac{\partial D}{\partial x} \frac{\partial[\text{IgG}]}{\partial x} \\
\frac{\partial[\text{IgG}]}{\partial t}_{\text{release}} &= \frac{\partial[\text{IgG}]}{\partial t}_{\text{total}} - \frac{\partial[\text{pAG}^{\text{MG}} \cdot \text{IgG} \cdot \text{dl5}]}{\partial[\text{IgG}]} \\
\frac{\partial[\text{IgG}]}{\partial t}_{\text{total}} &= \frac{\partial[\text{IgG}]}{\partial t}_{\text{diff}} / \left[1 - \frac{\partial[\text{pAG}^{\text{MG}} \cdot \text{IgG} \cdot \text{dl5}]}{\partial[\text{IgG}]} \right] \quad (5)
\end{aligned}$$

Zero-flux boundary conditions are enforced, and the total concentration of IgG within the vial is integrated and tracked throughout the simulation to estimate and control the error. The resulting model calculates the concentrations of both bound and unbound IgG with respect to time for various conditions and association constants and cooperativities. The apparent association constant K is estimated by assuming that the concentration of bound and unbound IgG within the hydrogel at $t = \infty$ are equal, and K is recalculated for each value of the Hill coefficient n from Eq. (6).

$$K = \frac{[\text{IgG} \cdot \text{dl5}]}{\left([\text{pAG}^{\text{MG}}] - [\text{IgG} \cdot \text{dl5}] \right) \cdot [\text{IgG}]_{\text{final}}^n} \quad (6)$$

2.9. Mice and in vivo studies

Six to eight week old female (certified-virus-free) BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the Duquesne University Animal Care Facility certified by the USDA. Animals were handled in accordance with protocols approved by Institutional Animal Care and Use Committee. The injection mixtures contained 60 nmol (in 20 μL) of EAK16-II together with 0.15 nmol of dL5_EAK, 0.15 nmol of pAG^{MG} , and 75 pmol of Dylight-800 4 $\times$ PEG conjugated IgG (Thermo Scientific). Mixtures, some without pAG^{MG} , were drawn into 25G' needled syringes and injected subcutaneously into the footpads of mice while anesthetized with isoflurane (induction 5%, maintenance 3%). For retention studies (Fig. 5), each mouse was injected with the system and control, but on different footpads. Four replicates were performed. For distribution studies (Fig. 6), each mouse received either solutions containing the intact system or control, in both footpads. Three replicates were performed for these experiments. In both studies, footpads and/or lymphatic organs were excised 72 h after injection and imaged using an Odyssey imager (Li-Cor, Inc., Lincoln, Nebraska). All images were captured at low intensity (L2.0) and the same focus offset (3.2 mm), and quantified using the software Image Studio (Li-Cor, Inc.). Table 1 shows the formulations used in the *in vivo* experiments.

3. Results

We developed a bioaffinity-driven system in which the release of IgG from a fibrillar matrix was retarded in order to concentrate therapeutic antibodies locally at injection sites and in regional lymph nodes. By concentrating antibodies in lymph nodes to which inflamed tissues drained, dosage of the drug injected can be reduced. Techniques of parental

injections targeting selected lymph nodes have been reported [41]. The current design hinges on engineering a fluorogenic module into the network of β FP within which binding sites for IgG can be monitored optically (Fig. 1a). Two dye-labeled IgG molecules, IgG^{FTIC} and IgG⁸⁰⁰, were used as model cargos for *in vitro* and *in vivo* studies, respectively. The polypeptide dL5_EAK does not coalesce, making it possible to recover from *E. coli* cell lysates using standard column purification methods. The identity of dL5_EAK was verified using SDS-PAGE and mass spectrometry [28]. Building upon the prototype described in Saunders et al. [28], we determined here that intermixing EAK16-II with dL5_EAK at 400:1 M ratio resulted in 94% of the latter being incorporated into the final composite (Fig. 1b). Unless noted otherwise, the experiments described herein were conducted using the same molar ratio.

To characterize the effect of mixing dL5_EAK and EAK16-II, the viscosities of solutions containing both were measured using capillary rheometry by which pressure gradient of the sample is measured across microfluidic channels [42]. Consistent with results reported by the Chen group [43], viscosity of diluted EAK16-II peaked at approximately 25% PBS (Supplemental material S2), indicating that the method is sensitive to the same material behavior of the β FP. Mixtures containing EAK16-II and dL5_EAK were tested in a range of shear rates at 25 °C in PBS. Solutions dissolved with only dL5_EAK exhibited baseline viscosity, at near 1 mPa·s (Fig. 1c), confirming our previous observations that the polypeptide does not self-assemble at the concentrations tested. At shear rate of 4000 s⁻¹, the viscosity values of EAK16-II and EAK16-II mixed with dL5_EAK both registered between 1 to 1.5 mPa·s, indicating that the materials lack shear resistance. Scanning EAK16-II in PBS at lower shear rates (1000–3000 s⁻¹) revealed a sharp transition at 1800 s⁻¹ for EAK16-II (Fig. 1c); the downward trend from 2.1 mPa·s to 1.5 mPa·s suggests a pseudo-elastic material. The transition was attenuated in the presence of dL5_EAK, with viscosity measured between 1.3 to 1.5 mPa·s within the range of shear rates tested. Due to the low concentrations of EAK16-II used and lack of resistance, the materials are thus characterized as films. Intermixing the two peptides does not diminish staining by Congo red [28]. These results show that the microscopic organization in EAK16-II appeared sensitive to perturbation by dL5_EAK, but the macroscopic structure appeared intact. The lack of mechanical strength of the materials is not unexpected because the low-molecular weight and the low concentration of the β FP used. The mixture can be injected without clogging, for the shear rate applied in 25 gauged syringe needles is estimated approximately 8000 s⁻¹. The deformable coacervates can adapt to tissue space when injected subcutaneously.

3.1. Localization of IgG in pAG^{MG}-embedded dL5 film

The conjugate pAG^{MG} was tested for binding to composites of dL5_EAK mixed with molar excess of EAK16-II (referred to as “dL5 film” hereafter) pre-formed on plastic surface. K_D between pAG^{MG} and dL5 gel was determined to be 90.9 nM, calculated based on titration experiments using MG fluorescence as the readout (Fig. 2a). Saturation was observed at molar ratio at 1:1 between pAG^{MG} and dL5. No fluorescence was observed in PBS containing pAG^{MG}, or in water dissolved with EAK16-II without dL5_EAK (Supplemental material S3a), all indicating specific binding of pAG^{MG} to dL5.

Using laser confocal scanning microscopy, we verified pAG^{MG} indeed mediated IgG loading in the dL5 film. We observed scattered, non-specific IgG adsorption (green) on the surface, but the extensive co-localization (yellow) underneath suggests dL5-bound pAG^{MG} captured the majority of the antibodies in the matrix (Fig. 2). Without pAG^{MG}, IgG^{FITC} in the matrix was significantly reduced, as evidenced by the relatively low fluorescence (Supplementary material S3b). These data support the fluorescence-generating IgG binding sites. We determined that at least 70 μg of IgG could be loaded per mg of the βFP peptides, although more could be loaded as the EAK16-II fibrillar network contains large aqueous void volume (99.5% w/v). Sub-saturating doses may suffice for delivering antibodies for local effects, for example with an anti-tumor necrosis factor antibody [44].

3.2. In vitro IgG release from pAG^{MG}-embedded dL5 film

Release of IgG^{FITC} from pAG^{MG}-embedded dL5 film was monitored in vitro over two months. The experiment was initiated by mixing the antibody with the rest of the peptides and proteins in a small cylindrical vessel with defined dimensions. In the control experiment in which films were made without pAG^{MG}, almost 90% (mean value, $n = 3$) of the antibody loaded were released in the first five days (Fig. 3a). In contrast, a biphasic pattern was seen with films embedded with pAG^{MG}; by day 5, 26% were released, followed by a second phase during which approximately 50% were released over 2 months. At the end of the eight-week period, 22.7% of the IgG still remained. The cumulative amount released might suffice the dosage needed for local antibody drug delivery [44]. The IgG^{FITC}, an anti-human IgG antibody, released from the films at the end of the 2-month period were shown to bind its cognate antigen (human IgG) at 58% capacity: 1.7 $\mu\text{g}/\text{mL}$ of the 2.9 $\mu\text{g}/\text{mL}$ anti-human IgG released recognized plastic-adsorbed human IgG (Supplemental material S4). Dissociation of pAG^{MG} from dL5 appeared slow, as evidenced by that more than 60% of the initial MG fluorescence remaining in the film at the end (Fig. 3b). These results demonstrate that the affinity binding delayed the release of IgG from the film, and that pAG^{MG}-bound dL5 is a relatively stable component.

3.3. Modeling and simulation of release

We sought to understand the parameters governing the kinetics of IgG release from the film. The initial phase of the *in vitro* release was fit to the non-steady state Fickian model (Fig. 4a and Table 2). Apparent diffusion coefficients (D_{app}) were determined by relating M_t/M_∞ as a function of $t^{0.5}$. D_{app} of IgG in films without pAG^{MG} was an order of magnitude higher than D_{app} of IgG in films embedded with pAG^{MG}. Release of IgG was impeded in both systems, as D_{app} of IgG in aqueous solution is estimated much higher [13]. While the analysis was not meant to encapsulate the entire release profile, the results show that without pAG^{MG}, a large fraction of loaded IgG would escape by rapid diffusion shortly after the components coalesce.

The numerical model outlined in Section 2.8 was used to further examine the experimental results, expanding on this observed decrease in apparent diffusion with the bioaffinity film. Fig. 4b shows the predicted evolution of the unbound IgG along the length of the vial, assuming 1-D diffusion. Initially all IgG is assumed to exist only in the films, and the concentration along the length of the vial is approximated through a sharp sigmoidal

function at the boundary of the fibrillar matrix. The diffusion coefficient is assumed to vary in a similar fashion between the Control value and the IgG in aqueous values in Table 2, and the difference between the measured Control and system values is accounted for by the binding behavior within the model. The concentration is calculated by dividing the initial IgG amount by the volume of the matrix, assuming an even distribution. Eq. (4) is then used to divide the concentration into bound and unbound concentrations. As the model IgG diffuses out of the coacervates into the release media, additional IgG is dissociated from pAG^{MG}, thereby replenishing in the matrix the IgG lost to diffusion.

For a comparison against Fig. 3b, the concentration of unbound IgG outside of the matrix region was integrated with respect to time and divided by the saturation concentration. This is calculated as the original number of moles of IgG within the vial divided by the total volume of the vial ($[IgG]_{final}^n$), and remains constant between each case. Four cases are recreated; one without pAG^{MG}, and three with varying levels of cooperativity between the binding sites on pAG^{MG}, $n = 0.5$ (negative cooperative binding), 1.0, and 2.0 (positive cooperative binding) as defined in Eqs. (4) and (6). The apparent association constant K is estimated by assuming that the concentration of bound and unbound IgG within the film at $t = \infty$ are equivalent, and K is recalculated for each value of the Hill coefficient n from Eq. (4). The values for the association constant K are calculated as 0.0251, 0.0496, and 0.1947 for $n = 0.5$, 1.0, and 2.0 respectively.

The molar concentration of IgG outside of the film is integrated across the length of the vial and divided by the equilibrium concentration, where the moles of IgG are evenly distributed across the vial volume. The total fluid volume considered is 250 μ L to better approximate the original fluid left in the vial between measurements. This methodology does not take into account the experimental removal and replenishment of the buffer solution, as this removal of IgG concentrations introduced undesirable instability in the finite difference method. Consequently the model under predicts the diffusion rate for the Control case, likely due to a combination of the removal/replenishment behavior combined with the potential convective flow introduced as a result. However, the complete system simulations in which IgG is bound to the fibrillar matrix provides a favorable result, indicating that the binding between IgG and pAG^{MG} is responsible for the controlled rate of diffusion, and suggesting that the nature of the binding is cooperative. If the diffusion for the complete system is enhanced experimentally by the removal/replenishment of the buffer solution, then the binding is likely to further favor the cooperative case.

Simulated cases containing a positive cooperativity show a more sustained release. Comparing the results to Fig. 3a, it is demonstrated that the improved and controlled release offered by the bioaffinity film may be predicted through a numerical model, and this model predicts that the IgG-pAG^{MG} binding is cooperative. The profile of the release is intrinsically tied to the hexavalent binding between IgG and pAG^{MG}, and this binding is crucial for the controlled and sustained release observed in experiments. From the plots given, the estimated values for the pAG^{MG}-IgG binding are an association constant of $K \approx 0.1947$, and a Hill coefficient of $n \approx 2.0$.

3.4. Deposition of dL5-film formulated IgG *in vivo*

Next we tested the hypothesis that dL5 film serves to retain IgG *in vivo*. To this end, dL5_EAK and EAK16-II mixed with pAG^{MG} and IgG⁸⁰⁰ were injected subcutaneously into footpads of mice and imaged *ex vivo* (Fig. 5a). The results indicate that the film and antibody localized beneath the skin (Fig. 5b). Compared to IgG⁸⁰⁰ formulated without pAG^{MG}, the intact film rendered superior retention at the injection site, as reflected by the relatively strong fluorescence emitted at the 800 nm channel (green). The concentrated fluorescence sustained for at least 72 h, after which the signals in both sets of mice dissipated to background level. Movement of animals would no doubt perturb the materials located in the subcutaneous space. To this end, we probed the localization of the pAG^{MG}-dL5 films in footpads of living mice. In a separate experiment, it was detected at the injection site (after 48 h) that IgG⁸⁰⁰ (green) co-localized with pAG^{MG}-bound dL5 (red; 700 nm) (Fig. 5c). No MG fluorescence was detected in the control footpad administered with free IgG⁸⁰⁰. Note that in footpads injected with the intact system, IgG was also detected in the draining lymph node (indicated by arrow). No MG fluorescence was detected in the same lymph node, consistent with the *in vitro* observation that the pAG^{MG}/dL5 complex is a stable component in the film. These results demonstrate consistent coalescence of IgG⁸⁰⁰, pAG^{MG}, dL5_EAK, and EAK16-II *in vivo*.

In a separate set of mice, distribution of IgG⁸⁰⁰ was traced based on fluorescence detected in regional and distant lymphoid organs (Fig. 6). Mice injected with the antibody in plain PBS (into both footpads) resulted in detectable fluorescence in sciatic, inguinal, brachial lymph nodes, and to a lesser extent, the spleen. Distribution of IgG⁸⁰⁰ delivered with the system (*i.e.* pAG^{MG}, dL5_EAK and EAK16-II) was mostly sequestered to sciatic lymph nodes, draining from the footpads; inguinal and brachial nodes showed low fluorescence, with spleen at near background level of intensity. In mice receiving the intact system, the low fluorescence in distant lymph nodes is congruent with the strong fluorescence remaining at the injection site. These results indicate the dL5 film impeded the distribution of IgG deposited at the injection site.

4. Discussion

This work was motivated by the need for a trackable injectable system by which antibodies could be concentrated *in vivo*. Potential applications include intratumoral antibody injection [29], and topical delivery of anti-cytokine antibodies [44]. The inflammatory mediator TNF α driving non-healing cutaneous wound is a case in point. In addition to releasing soluble TNF α , macrophages infiltrated into the wound tissues express transmembrane TNF α [45]. Together with neutrophils and adipocytes, macrophages impose local inflammation *via* autocrine and paracrine actions of both forms of TNF α . To maximize effectiveness, topically delivered anti-TNF antibody must penetrate into tissues underneath the wound bed. Macrophages and neutrophils transverse on and below the wound surface, trafficking through the dermis and draining lymph nodes, and cluster around adipocytes underneath. Thus design of topical formulations should aim at rendering sustained anti-cytokine antibodies releasing into local-regional tissues, yet without excessive drug partitioning into the blood circulation.

The results demonstrate that the dL5 film can accumulate IgG at the site of injection and in draining lymph nodes. The study is a step towards modular engineering of local antibody delivery. The binding of pAG^{MG} to dL5 serves two purposes, in rendering IgG binding sites, and generating fluorescence that can be detected in subcutaneous tissues. The system delayed the release of IgG; *in vitro*, approximately 50% of IgG^{FITC} was released in the last 55 days of the two-month period with dL5 films embedded with pAG^{MG}, without which near 90% of the antibody was released in the first five days (Fig. 3). The adaptor pAG^{MG} mediated IgG loading (Fig. 2), and was found to be a relatively immobile component in the film (Fig. 3). *In vivo*, distribution of IgG was impeded by the dL5 film, restricting a large fraction of the dose at the injection site and in the immediate draining lymph nodes (Figs. 5 and 6). The prototype delivered a limited dose over a 24-h period, but such formulation may be sufficed for local immune modulation [44]. The computational simulations suggest cooperative binding of IgG to pAG^{MG} (Fig. 4). The film is generated through coassembly for functionalization of β FP [38,46–49]. We have shown that the EAK16-II/EAKIIIH6 composite prolongs *in vivo* retention of IgG in inflammatory tissues, in mammary tumors implanted in syngeneic mice [29], and in tissues underneath skin allografts [30]. By separating affinity binding and coacervation, the former can be adjusted independently of the latter. The ability to vary the density of dL5 through intermixing the polypeptides allows mutual optimization of bulk physical properties and IgG loading and release kinetics. Because the pore size and tortuosity of the fibrillar matrix can be tuned by changing EAK16-II concentration, other agents, including small molecule drugs, or clusters of cells [50], may be encapsulated without impeding the ability to regulate IgG loading and release. In the presence of molar excess EAK16-II, dL5_EAK incorporates efficiently into the network, without disrupting the integrity of the fibrils [28]. But excess dL5_EAK *in vivo* may self-aggregate (similar to EAKH6 [10]), creating ectopic clusters of pAG^{MG} outside the film, thereby diverting IgG from the main depot. This can be mitigated by formulating the molar fractions of the two polypeptides such greater than 90% of dL5_EAK are incorporated (Fig. 1). Thus the ratio of the two polypeptides is a critical parameter in engineering functionalities into the EAK16-II fibrillar matrix [51].

Over a period of two months *in vitro*, IgG released from dL5 films retained 58% of antigen-binding capacity (Supplemental material S4). The high water content of EAK16-II films should preserve protein native conformations [52], but the long incubation period *in vitro* (Fig. 3) may have promoted denaturation of the IgG. Through Fc binding to pAG^{MG}, IgG in dL5 films are oriented [53–55] and patterned such that random adsorption and denaturation can be reduced. This is supported by scanning electronic microscopic images of EAK16-II/EAKIIIH6, which show what appear as individual islands of IgG/pAG complexes [29]. The templated antibodies are active against soluble [29] and cell-bound antigens [31]. Denaturation can be minimized by optimizing key parameters; the simulations (Fig. 4) show cooperativity of IgG binding in the system driven by the hexavalent pAG^{MG} on which two or more IgG may be captured. Altering the density of pAG^{MG} in the films, guided by MG fluorescence, could minimize the loss of protein activity.

Potential systemic toxicities are mitigated by that the film is largely retained locally at the injection site. Because pAG^{MG} remained largely with the film, the amount released into the systemic lymphatic and blood circulation should be low. Phagocytes will likely opsonize the

fluorogen–protein conjugate. We have evidences showing that EAK16-II does not trigger acute inflammation; no signs of irritation or swelling was observed in footpads injected with the materials (Supplemental material S5), and neither EAK16-II nor pAG^{MG} elicited IL-6 production from freshly isolated splenocytes cultured *in vitro* (Supplemental material S6). As with other synthetic biomaterials, the film is likely be removed in part by the foreign body reaction (FBR) [56]. The mechanism is triggered by adsorption of endogenous proteins, followed by adhesion of macro-phages, which may fuse into foreign body giant cells (FBGC), which takes weeks to consummate.

The biocompatibility of the materials is not necessarily more complex than formulations of chimeric or humanized recombinant proteins. Infliximab, a chimeric mouse-human IgG approved by the US Food and Drug Administration and the European Medicines Agency over a decade ago, can trigger anti-drug antibodies in as many as 44% of patients [57], but such immune responses has not precluded its clinical use [58]. Anti-drug antibodies have been documented in patients receiving adalimumab [59], a humanized antibody, which remains one of the most prescribed biologic. The *Staphylococcal* Protein A (SpA) has been studied in humans as an immune modulator (clinical trials.gov: NCT01749787 and NCT00517855). Two additional clinical trials are ongoing (NCT02330445 and NCT02401061); up to 420 mcg may be administered in five weeks. The data so far indicate, PRTX-100, a purified form of SpA, is safe and well-tolerated in humans [33]. While anti-SpA antibodies have been detected, they neither preclude the biological actions nor elicit serious adverse reactions. Such bystander antibodies are not uncommon in biologic drugs. In 2015, PRTX-100 received orphan drug designation from the FDA. Thus pAG is not necessarily more immunogenic than some of the recombinant biologics currently used in humans [60]. Analysis of risks and benefits may justify the use of pAG in formulation development in intractable problems such as non-healing cutaneous wounds.

The system described herein is unique in that the bioaffinity module has a built-in imaging feature. In dL5-pAG^{MG}, the film can be imaged in superficial tissues. Compared to conventional fluorescent reporters, MG-dL5 has enhanced photo-stability. The FAP protects the fluorogen from oxidative damage as crystallographic studies show that a single MG molecule is flanked by two L5 domains [35]. This feature was exploited here to monitor the stability of the IgG binding sites, as pAG^{MG} would escape from the film matrix to become non-fluorescent. Degradation of dL5, either through extracellular or intracellular means, would diminish the signal, as unbound MG has low intrinsic fluorescence. The fluorescence generated by MG and dL5 interaction could serve as a surrogate for the extent of *in vivo* degradation, such as phagocytosis by macrophages [61].

While the present study used dL5-MG as the fluorogenic module, other FAPs have been synthesized for additional fluorogens. FAP-fluorogen pairs can be selected based on binding affinities and fluorescent spectra suited for specific applications. Fluorescence generated from dL5-MG can be detected optically in superficial tissues, but other FAP systems, such as far-red fluorogenic dyes currently being explored, may be used for internal imaging [62]. There are also promiscuous FAPs that can bind more than one fluorogen [63], with alternate excitation and emission wavelengths and varying affinity constants for fluorogen binding. There may be biological scenarios in which higher or lower binding affinities are called for,

such as using a low affinity FAP-fluorogen combination as a more rapid but controlled release mechanism, through disassociation of the pAG-dye conjugate from the FAP itself. Additionally, other dyes which generate higher quantum yields and signal such as cyanine-tagged MG [64] could be used to improve signal-to-noise ratio for film with low FAP incorporation efficiency. These other FAPs can be genetically fused with β FP domains such as AEAEAK AK as functional modules in excess EAK16-II, as demonstrated here.

5. Conclusions

The results demonstrate biochemical and imaging functionalization of a β FP-based injectable platform. The depot platform can be used to deliver IgG locally of a variety of specificities. The delayed release demonstrated *in vitro* might be translated into *in vivo* prototypes through iterative optimization, guided by *in situ* detection of FAP-generated fluorescence. The design could be used to deliver antibodies for suppressing cutaneous inflammation and targeted immune modulation in draining lymph nodes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by a Health Research Formula award from the Pennsylvania Department of Health (W.S.M.), and National Institutes of Health grants R21AI113000 (W.S.M) and U54GM10329 (A.S.W.).

References

1. Vacchelli E, Pol J, Bloy N, Eggermont A, Cremer I, Fridman WH, Galon J, Marabelle A, Kohrt H, Zitvogel L, Kroemer G, Galluzzi L. Trial watch: tumor-targeting monoclonal antibodies for oncological indications. *Oncoimmunology*. 2015; 4:e985940. [PubMed: 25949870]
2. Bhattarai N, Gunn J, Zhang M. Chitosan-based hydrogels for controlled, localized drug delivery. *Adv Drug Deliv Rev*. 2010; 62:83–99. [PubMed: 19799949]
3. Hotelling NA, Tang L, Irvine DJ, Babensee JE. Biomaterial Strategies for Immunomodulation. *Annu Rev Biomed Eng*. 2015
4. Cruise GM, Scharp DS, Hubbell JA. Characterization of permeability and network structure of interfacially photopolymerized poly(ethylene glycol) diacrylate hydrogels. *Biomaterials*. 1998; 19:1287–1294. [PubMed: 9720892]
5. Jeong B, Bae YH, Kim SW. Drug release from biodegradable injectable thermosensitive hydrogel of PEG-PLGA-PEG triblock copolymers. *J Control Release*. 2000; 63:155–163. [PubMed: 10640589]
6. Lin CC, Metters AT. Hydrogels in controlled release formulations: network design and mathematical modeling. *Adv Drug Deliv Rev*. 2006; 58:1379–1408. [PubMed: 17081649]
7. Zhang S, Holmes T, Lockshin C, Rich A. Spontaneous assembly of a self-complementary oligopeptide to form a stable macroscopic membrane. *Proc Natl Acad Sci U S A*. 1993; 90:3334–3338. [PubMed: 7682699]
8. Zhang S. Fabrication of novel biomaterials through molecular self-assembly. *Nat Biotechnol*. 2003; 21:1171–1178. [PubMed: 14520402]
9. Keyes-Baig C, Duhamel J, Fung SY, Bezaire J, Chen P. Self-assembling peptide as a potential carrier of hydrophobic compounds. *J Am Chem Soc*. 2004; 126:7522–7532. [PubMed: 15198599]
10. Wen Y, Roudebush SL, Buckholtz GA, Goehring TR, Giannoukakis N, Gawalt ES, Meng WS. Coassembly of amphiphilic peptide EAK16-II with histidinylated analogues and implications for

functionalization of β -sheet fibrils in vivo. *Biomaterials*. 2014; 35:5196–5205. [PubMed: 24680662]

11. Emamyari S, Fazli H. pH-dependent self-assembly of EAK16 peptides in the presence of a hydrophobic surface: coarse-grained molecular dynamics simulation. *Soft Matter*. 2014; 10:4248–4257. [PubMed: 24740580]
12. Koutsopoulos S, Unsworth LD, Nagai Y, Zhang S. Controlled release of functional proteins through designer self-assembling peptide nanofiber hydrogel scaffold. *Proc Natl Acad Sci U S A*. 2009; 106:4623–4628. [PubMed: 19273853]
13. Koutsopoulos S, Zhang S. Two-layered injectable self-assembling peptide scaffold hydrogels for long-term sustained release of human antibodies. *J Control Release*. 2012; 160:451–458. [PubMed: 22465676]
14. Sakiyama-Elbert SE, Hubbell JA. Controlled release of nerve growth factor from a heparin-containing fibrin-based cell ingrowth matrix. *J Control Release*. 2000; 69:149–158. [PubMed: 11018553]
15. Sakiyama-Elbert SE, Hubbell JA. Development of fibrin derivatives for controlled release of heparin-binding growth factors. *J Control Release*. 2000; 65:389–402. [PubMed: 10699297]
16. Taylor SJ, Mc Donald JW 3rd, Sakiyama-Elbert SE. Controlled release of neurotrophin-3 from fibrin gels for spinal cord injury. *J Control Release*. 2004; 98:281–294. [PubMed: 15262419]
17. Wood MD, Sakiyama-Elbert SE. Release rate controls biological activity of nerve growth factor released from fibrin matrices containing affinity-based delivery systems. *J Biomed Mater Res A*. 2008; 84:300–312. [PubMed: 17607752]
18. Wood MD, Hunter D, Mackinnon SE, Sakiyama-Elbert SE. Heparin-binding-affinity-based delivery systems releasing nerve growth factor enhance sciatic nerve regeneration. *J Biomater Sci Polym Ed*. 2010; 21:771–787. [PubMed: 20482984]
19. Sakiyama-Elbert SE. Incorporation of heparin into biomaterials. *Acta Biomater*. 2014; 10:1581–1587. [PubMed: 24021232]
20. Soontornworajit B, Zhou J, Zhang Z, Wang Y. Aptamer-functionalized in situ injectable hydrogel for controlled protein release. *Biomacromolecules*. 2010; 11:2724–2730. [PubMed: 20809645]
21. Soontornworajit B, Zhou J, Snipes MP, Battig MR, Wang Y. Affinity hydrogels for controlled protein release using nucleic acid aptamers and complementary oligonucleotides. *Biomaterials*. 2011; 32:6839–6849. [PubMed: 21684002]
22. Battig MR, Soontornworajit B, Wang Y. Programmable release of multiple protein drugs from aptamer-functionalized hydrogels via nucleic acid hybridization. *J Am Chem Soc*. 2012; 134:12410–12413. [PubMed: 22816442]
23. Battig MR, Huang Y, Chen N, Wang Y. Aptamer-functionalized superporous hydrogels for sequestration and release of growth factors regulated via molecular recognition. *Biomaterials*. 2014; 35:8040–8048. [PubMed: 24954732]
24. Vulic K, Shoichet MS. Tunable growth factor delivery from injectable hydrogels for tissue engineering. *J Am Chem Soc*. 2012; 134:882–885. [PubMed: 22201513]
25. Vulic K, Pakulska MM, Sonthalia R, Ramachandran A, Shoichet MS. Mathematical model accurately predicts protein release from an affinity-based delivery system. *J Control Release*. 2015; 197:69–77. [PubMed: 25449806]
26. Vulic K, Shoichet MS. Affinity-based drug delivery systems for tissue repair and regeneration. *Biomacromolecules*. 2014; 15:3867–3880. [PubMed: 25230248]
27. Pakulska MM, Vulic K, Shoichet MS. Affinity-based release of chondroitinase ABC from a modified methylcellulose hydrogel. *J Control Release*. 2013; 171:11–16. [PubMed: 23831055]
28. Saunders MJ, Liu W, Szent-Gyorgyi C, Wen Y, Drennen Z, Waggoner AS, Meng WS. Engineering fluorogen activating proteins into self-assembling materials. *Bioconjug Chem*. 2013; 24:803–810. [PubMed: 23573960]
29. Wen Y, Kolonich HR, Kruszewski KM, Giannoukakis N, Gawalt ES, Meng WS. Retaining antibodies in tumors with a self-assembling injectable system. *Mol Pharm*. 2013; 10:1035–1044. [PubMed: 23419203]

30. Wen Y, Liu W, Bagia C, Zhang S, Bai M, Janjic JM, Giannoukakis N, Gawalt ES, Meng WS. Antibody-functionalized peptidic membranes for neutralization of allogeneic skin antigen-presenting cells. *Acta Biomater.* 2014; 10:4759–4767. [PubMed: 25117952]
31. Zheng Y, Wen Y, George AM, Steinbach AM, Phillips BE, Giannoukakis N, Gawalt ES, Meng WS. A peptide-based material platform for displaying antibodies to engage T cells. *Biomaterials.* 2011; 32:249–257. [PubMed: 20880580]
32. Saha K, Bender F, Gizeli E. Comparative study of IgG binding to proteins G and A: nonequilibrium kinetic and binding constant determination with the acoustic waveguide device. *Anal Chem.* 2003; 75:835–842. [PubMed: 12622374]
33. Ballow C, Leh A, Slentz-Kesler K, Yan J, Haughey D, Bernton E. Safety, pharmacokinetic, immunogenicity, and pharmacodynamic responses in healthy volunteers following a single intravenous injection of purified staphylococcal protein A. *The Journal of Clinical Pharmacology.* 2013; 53:909–918. [PubMed: 23832863]
34. Szent-Gyorgyi C, Schmidt BF, Creeger Y, Fisher GW, Zakel KL, Adler S, Fitzpatrick JA, Woolford CA, Yan Q, Vasilev KV, Berget PB, Bruchez MP, Jarvik JW, Waggoner A. Fluorogen-activating single-chain antibodies for imaging cell surface proteins. *Nat Biotechnol.* 2008; 26:235–240. [PubMed: 18157118]
35. Szent-Gyorgyi C, Stanfield RL, Andreko S, Dempsey A, Ahmed M, Capek S, Waggoner AS, Wilson IA, Bruchez MP. Malachite green mediates homodimerization of antibody VL domains to form a fluorescent ternary complex with singular symmetric interfaces. *J Mol Biol.* 2013; 425:4595–4613. [PubMed: 23978698]
36. Fu AS, Thatiparti TR, Saidel GM, von Recum HA. Experimental studies and modeling of drug release from a tunable affinity-based drug delivery platform. *Ann Biomed Eng.* 2011; 39:2466–2475. [PubMed: 21678091]
37. Hoffman, JD., Frankel, S. *Numerical Methods for Engineers and Scientists.* CRC press; 2001.
38. Hill AV. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *J Physiol (Lond).* 1910; 40:4–7.
39. Grove TL, Baker MB. Phase equilibrium controls on the tholeiitic versus calcalkaline differentiation trends. *Journal of Geophysical Research: Solid Earth.* 1984; 89:3253–3274. 1978–2012.
40. Baker MB, Grove TL. Kinetic controls on pyroxene nucleation and metastable liquid lines of descent in a basaltic andesite. *Am Mineral.* 1985; 70:279–287.
41. Engman C, Wen Y, Meng WS, Bottino R, Trucco M, Giannoukakis N. Generation of antigen-specific Foxp3+ regulatory T-cells in vivo following administration of diabetes-reversing tolerogenic microspheres does not require provision of antigen in the formulation. *Clin Immunol.* 2015
42. Zarraga IE, Taing R, Zarzar J, Luoma J, Hsiung J, Patel A, Lim FJ. High shear rheology and anisotropy in concentrated solutions of monoclonal antibodies. *J Pharm Sci.* 2013; 102:2538–2549. [PubMed: 23873347]
43. Jun S, Hong Y, Imamura H, Ha BY, Bechhoefer J, Chen P. Self-assembly of the ionic peptide EAK16: the effect of charge distributions on self-assembly. *Biophys J.* 2004; 87:1249–1259. [PubMed: 15298927]
44. Friedrich EE, Azofiefa A, Fisch E, Washburn NR. Local delivery of antitumor necrosis factor- α through conjugation to hyaluronic acid: dosing strategies and early healing effects in a rat burn model. *Journal of Burn Care & Research.* 2015; 36:e90–e101. [PubMed: 25526179]
45. Xu H, Uysal KT, Becherer JD, Arner P, Hotamisligil GS. Altered tumor necrosis factor- α (TNF- α) processing in adipocytes and increased expression of transmembrane TNF- α in obesity. *Diabetes.* 2002; 51:1876–1883. [PubMed: 12031976]
46. Mahmoud ZN, Grundy DJ, Channon KJ, Woolfson DN. The non-covalent decoration of self-assembling protein fibers. *Biomaterials.* 2010; 31:7468–7474. [PubMed: 20638122]
47. Collier JH, Rudra JS, Gasiorowski JZ, Jung JP. Multi-component extracellular matrices based on peptide self-assembly. *Chem Soc Rev.* 2010; 39:3413–3424. [PubMed: 20603663]

48. Jung JP, Moyano JV, Collier JH. Multifactorial optimization of endothelial cell growth using modular synthetic extracellular matrices. *Integr Biol (Camb)*. 2011; 3:185–196. [PubMed: 21249249]
49. Gelain F, Silva D, Caprini A, Taraballi F, Natalello A, Villa O, Nam KT, Zuckermann RN, Doglia SM, Vescovi A. BMHP1-derived self-assembling peptides: hierarchically assembled structures with self-healing propensity and potential for tissue engineering applications. *ACS Nano*. 2011; 5:1845–1859. [PubMed: 21314189]
50. Tajima A, Liu W, Pradhan I, Bertera S, Bagia C, Trucco M, Meng WS, Fan Y. Bio-engineering mini functional thymic units with EAK16-II/EAKIIH6 self-assembling hydrogel. *Clin Immunol*. 2015
51. Wen Y, Roudebush SL, Buckholtz GA, Goehring TR, Giannoukakis N, Gawalt ES, Meng WS. Coassembly of amphiphilic peptide EAK16-II with histidinylated analogues and implications for functionalization of beta-sheet fibrils in vivo. *Biomaterials*. 2014; 35:5196–5205. [PubMed: 24680662]
52. Annabi N, Tamayol A, Uquillas JA, Akbari M, Bertassoni LE, Cha C, Camci-Unal G, Dokmeci MR, Peppas NA, Khademhosseini A. 25th anniversary article: rational design and applications of hydrogels in regenerative medicine. *Adv Mater*. 2014; 26:85–123. [PubMed: 24741694]
53. Leckband DE. The influence of protein and interfacial structure on the self-assembly of oriented protein arrays. *Adv Biophys*. 1997; 34:173–190. [PubMed: 9204134]
54. Vijayendran RA, Leckband DE. A quantitative assessment of heterogeneity for surface-immobilized proteins. *Anal Chem*. 2001; 73:471–480. [PubMed: 11217749]
55. Johnson CP, Jensen IE, Prakasam A, Vijayendran R, Leckband D. Engineered protein a for the orientational control of immobilized proteins. *Bioconjug Chem*. 2003; 14:974–978. [PubMed: 13129401]
56. Bryers JD, Giachelli CM, Ratner BD. Engineering biomaterials to integrate and heal: the biocompatibility paradigm shifts. *Biotechnol Bioeng*. 2012; 109:1898–1911. [PubMed: 22592568]
57. Bendtzen K, Geborek P, Svenson M, Larsson L, Kapetanovic MC, Saxne T. Individualized monitoring of drug bioavailability and immunogenicity in rheumatoid arthritis patients treated with the tumor necrosis factor alpha inhibitor infliximab. *Arthritis Rheum*. 2006; 54:3782–3789. [PubMed: 17133559]
58. Baert F, Noman M, Vermeire S, Van Assche G, DH G, Carbonez A, Rutgeerts P. Influence of immunogenicity on the long-term efficacy of infliximab in Crohn's disease. *The New England Journal of Medicine*. 2003; 348:601–608. [PubMed: 12584368]
59. Bartelds GM, Krieckaert CL, Nurmohamed MT, van Schouwenburg PA, Lems WF, Twisk JW, Dijkmans BA, Aarden L, Wolbink GJ. Development of antidrug antibodies against adalimumab and association with disease activity and treatment failure during long-term follow-up. *JAMA: The Journal of the American Medical Association*. 2011; 305:1460–1468. [PubMed: 21486979]
60. Garcês S, Demengeot J, Benito-Garcia E. The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systematic review of the literature with a meta-analysis. *Ann Rheum Dis*. 2013; 72:1947–1955. [PubMed: 23223420]
61. Salvador-Morales C, Zhang L, Langer R, Farokhzad OC. Immunocompatibility properties of lipid-polymer hybrid nanoparticles with heterogeneous surface functional groups. *Biomaterials*. 2009; 30:2231–2240. [PubMed: 19167749]
62. Zhang M, Chakraborty SK, Sampath P, Rojas JJ, Hou W, Saurabh S, Thorne SH, Bruchez MP, Waggoner AS. Fluoromodule-based reporter/probes designed for in vivo fluorescence imaging. *J Clin Invest*. 2015; 125:3915–3927. [PubMed: 26348895]
63. Ozhalici-Unal H, Pow CL, Marks SA, Jesper LD, Silva GL, Shank NI, Jones EW, Burnette JM 3rd, Berget PB, Armitage BA. A rainbow of fluoromodules: a promiscuous scFv protein binds to and activates a diverse set of fluorogenic cyanine dyes. *J Am Chem Soc*. 2008; 130:12620–12621. [PubMed: 18761447]
64. Szent-Gyorgyi C, Schmidt BF, Fitzpatrick JA, Bruchez MP. Fluorogenic dendrons with multiple donor chromophores as bright genetically targeted and activated probes. *J Am Chem Soc*. 2010; 132:11103–11109. [PubMed: 20698676]

Fig. 1.

Co-assembly and association of system components. (a) Schematic depiction of the affinity film for release of IgG. (b) Analysis of incorporation of dL5_EAK into EAK16-II fibrils using SDS-PAGE. Increasing amounts of EAK16-II were added to the same amount of dL5_EAK to make the ratio of the two from 50:1–800:1. The bands represent unincorporated, soluble fraction in the mixture after centrifugation. (c) Viscosity of EAK16-II mixed with dL5_EAK at different shear rates ($n = 3$). Measurements were made at 25 °C using a μ VISC unit equipped with a temperature control module.

Fig. 2. Binding and localization of pAG^{MG} in dL5 film. (a) Titration of pAG^{MG} to established films ($n = 3$). K_D was determined using the one site-specific binding model (y axis title: FI, fluorescent intensity; a.u., arbitrary unit). No washing steps were needed because no background fluorescence was generated from unbound MG. (b) Top view of IgG^{FITC}-loaded pAG^{MG}-dL5 films, the intact system, showing fluorescence associated with IgG (green), MG-dL5 (red), and the two combined. Scale bar represents 20 μm . (c) Side view of the intact systems, showing fluorescent components along the vertical axis of the gel. Gels were deposited into glass bottom microwells immediately prior to imaging. Controls examined included films made with components used for making the intact system without pAG^{MG} or dL5_EAK (Supplemental material, S3b).

Fig. 3.

Monitoring in vitro IgG release and pAG^{MG}-dL5 interaction. (a) Cumulative release of IgG^{FITC} from dL5 films with (n = 3) or without pAG^{MG} (n = 3). Each time point represents measurement from triplicate samples in films setup in parallel. (b) Images and quantification showing MG fluorescence in release vessels at the start and at the end of the period (n = 3 for all groups). “Start” refers to day 0, “End” refers to day 60, “Control” was the same in (a), and “Blank medium” refers to vials containing PBS with no other components added.

Fig. 4.

Modeling and simulation of IgG release *in vitro*. (a) Short time approximation of release of IgG^{FITC} *in vitro* (see Fig. 3) using Fick's second law. Because the small vial diameter relative to the vial length, the release is assumed 1-D along the vial length into the saline medium. No IgG escapes the sides and bottom of from the gel placed in the cylindrical glass vials. The volume of the gels was assumed constant, as β FP matrices do not swell. IgG was assumed distributed in the matrices homogenously and released in one-dimensionally, with IgG concentration at boundary assumed zero at the start. The height of the gel is 3.7 mm, with the base inner diameter equals to 4.15 mm (per manufacturer's specification). (b) The evolution of unbound IgG with respect to time for non-cooperative binding ($n = 1.0$). Initially the IgG is contained exclusively within the fibrillar matrix. Upon exposure to the release media, the IgG begins to diffuse into the simulated vial, accompanied by a gradual release of the bound IgG from the film. (c) The percentage of IgG released into the vial is plotted with respect to time with varied binding characteristics through the Hill equation. The release profiles show the effect of the binding characteristics, and an increase in sustained release achievable through cooperative hexavalent binding.

Fig. 5.

Retention of IgG and binding sites in footpad. Retention of IgG delivered with dL5 film at injection site. (a) Diagram depicting the *in vivo* experiments. Each mouse was injected subcutaneously with solutions containing all film components (system) in one footpad and the same except without pAG^{MG} (control) in the other. The mixture was drawn into a 25' G needled syringe as a low viscosity liquid by keeping in low ionic strength buffer. The mice were sacrificed at 72 h after the injection. (b) Images of four replicates showing fluorescence associated with IgG⁸⁰⁰ in footpads of each mouse three days after injection. (c) Images taken from a separate experiment 48 h after injection, showing fluorescence in mouse injected with IgG⁸⁰⁰ injected in saline (left footpad) and the same antibody injected in the same mouse with all components in the system (right footpad); the left panel shows the localization of IgG⁸⁰⁰, and right panel shows the location of pAG^{MG}-dL5 film (red). Injection of pAG^{MG} alone did not yield fluorescence above background level (Supplemental material S5).

Fig. 6. Distribution of IgG⁸⁰⁰ injected in footpads 72 h after injection. (a) Mice were injected with either control or system mixtures in both footpads. Unlike the experiments described in Fig. 5, here each mouse was injected with the same formulation. (b) The images show fluorescence associated with IgG⁸⁰⁰ in (1 and 1') sciatic, (2 and 2') inguinal, (3 and 3') brachial lymph nodes, the spleen, and footpads from mice administered with the intact film system mixed with IgG⁸⁰⁰, or IgG⁸⁰⁰ in PBS (control).

Table 1Formulations used in *in vivo* experiments.

	Intact system ^a	Control: retention ^b (μg)	Control: distribution ^c
EAK16-II (μg)	100	100	-
dL5_EAK (μg)	4.3	4.3	-
pAG ^{MG} (μg)	7.7	-	-
IgG (μg)	11.3 fluorescein	11.3 fluorescein	11.3 fluorescein
Total volume (μL)	49.4	49.4	49.4

^a“System” formulation used in experiments shown in Figs. 5 and 6.^b“Control” formulation used in experiments shown in Fig. 5.^c“Control” formulation used in experiments shown in Fig. 6.

Table 2

Apparent diffusivities (D_{app}) determined using approximation of early release^a.

	D_{app} (10^{-10} m ² s ⁻¹)	R ²	IgG remained in gel (%)
Control	0.075	0.92	1.4
System	0.0086	0.82	22.7
IgG in aq.	0.40 ^b	N/A	N/A

$$\frac{M_t}{M_\infty} = \left(\frac{16 D_{app} t}{\pi H^2} \right)^{0.5}$$

^aFick's second law was used in the approximation, with D_{app} being apparent diffusion coefficient, t as time in hours, H is one-dimensional height of matrix (3.7 mm), M_t being the amount of IgG released at time = t , and M_∞ being the theoretical maximal amount released at time infinity. D_{app} was determined by regression of the data to the equation:

^bCalculated based on the Stokes–Einstein approximation of IgG in water at 37 °C [13].