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(54) **Title**: MULTI-LAYERED INJECTABLE SELF-ASSEMBLING PEPTIDE SCAFFOLD HYDROGELS FOR LONG-TERM SUSTAINED RELEASE OF HUMAN ANTIBODIES

(57) **Abstract**: The invention relates to a pharmaceutical formulation for sustained delivery of a therapeutic agent, preferably a protein, polypeptide, an antibody or an antibody fragment, comprising one or more gel forming peptides wherein the formulation exhibits sustained delivery for at least two weeks, three weeks, four weeks, five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more. In one embodiment, the invention relates to a formulation comprising self-assembling peptides that undergo sol-gel transition in the presence of an electrolyte solution such as biological fluids and salts. The formulation can provide sustained release of antibody and antibody fragments, in particular, IgG. Antibody diffusivities can be decreased with increasing hydrogel nanofiber density, providing a means to control the release kinetics.

MULTI-LAYERED INJECTABLE SELF-ASSEMBLING PEPTIDE SCAFFOLD HYDROGELS FOR LONG-TERM SUSTAINED RELEASE OF HUMAN ANTIBODIES

RELATED APPLICATION

This application claims the benefit of U.S. Provisional Application No. 61/782,791, filed on March 14, 2013. The entire teachings of the above application are incorporated herein by reference.

BACKGROUND OF THE INVENTION

The use of hydrogels as drug delivery carriers has been explored since the beginning of the controlled release era in the 1960's primarily focusing on polymer hydrogels and animal-derived biomaterials. However, hydrogels consisting of synthetic polymers do not represent an ideal system for biomedical applications due to: (i) component and degradation product toxicity (e.g., many polymers require the use of toxic cross-linkers, like glutaraldehyde, and other chemicals that pose a life threat whereas others such as polyglycolic-polylactic acid and its analogues during degradation release acids locally), (ii) post-gelation polymer swelling often causes pain in the host, and (iii) release of the active compound over brief periods of time due to the large pores of the polymer network. Furthermore, animal-extracted biopolymers such as collagen, gelatin, fibrin, and laminin [1,2,3,4] are not considered in real-life, clinical applications involving humans due to their origin and the risk of inflammatory host response from viruses, bacteria, and other unknown substances that may be present in the donor tissue. In response to the need of biocompatible drug release systems, biodegradable synthetic polymers were developed [5,6,7,8]. Despite the

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extensive research and the constant development of novel hydrogel systems, these challenges have not been completely resolved yet.

Previously, a nanofiber hydrogel consisting of the self-assembling peptide ac-$(RADA)_4$-$CONH_2$ (where R is arginine, A is alanine and D is aspartic acid) was studied for controlled release of small, model-drug molecules [9]. In a recent study, it was shown that proteins with different molecular weights and isoelectric points were slowly released through the ac-$(RADA)_4$-$CONH_2$ peptide hydrogel and the release kinetics were studied over a period of 3 months[10]. Self-assembling peptide hydrogels are injectable because they can be formed inside the body upon interaction of the peptide solution with biological fluids. Upon being introduced to electrolyte solutions, self-assembling peptides form nanofibers with diameters between 10nm – 20nm, which are further organized to form a scaffold hydrogel containing water up to ~99.5% (w/v) and form pore with sizes between 5nm–200nm in diameter [11]. Peptide gelation does not require harmful materials, such as toxic cross-linkers, to initiate the sol-gel transition while the degradation products of the hydrogel are natural amino acids, which can be metabolized and reused by the body. The fact that the sol-gel transition occurs at physiological conditions facilitates mixing of the peptide solution with bioactive molecules and co-injection in a tissue-specific manner to form the drug delivery vehicle in the tissue. Peptide scaffold hydrogels are biocompatible, amenable to molecular design, and have been used in a number of tissue engineering applications including bone and cartilage reconstruction, neuronal and heart tissue regeneration, wound healing, angiogenesis, and haemostasis [12,13]. Self-assembling peptide hydrogels provide a platform that makes them

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ideal for a wide range of bionanomedical applications as they facilitate cell migration inside the hydrogel. Futhermore they are non-toxic, non-immunogenic, non-thrombogenic, biodegradable, and applicable to localized therapies through injection to a particular tissue [14,15].

Large proteins such as antibodies are larger and more complex than traditional organic and inorganic drugs due to the presence of multiple functional groups in addition to complex three-dimensional structures, and their formulation for sustained release poses difficult challenges. In order for the antibody to remain biologically active the formulation must protect the functional properties of the antibody for the duration of the therapy. There are multiple pathways for the antibody to degrade during sustained release due to the loss of three dimensional structure or chemical instability. These challenges are attenuated by the length of the therapy. The presentation of functional antibodies with therapeutic properties is important for sustained delivery biomedical applications. As such, there is a need to develop a sustained release system based on biodegragable peptides that can provide delivery of therapeutically useful large proteins such as an antibody for extended periods of time.

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SUMMARY OF THE INVENTION

The invention relates to a pharmaceutical formulation for sustained delivery of a therapeutic agent, preferably a protein, polypeptide, an antibody or an antibody fragment, comprising one or more gel forming peptides wherein the formulation exhibits sustained delivery for at least two weeks, three weeks, four weeks, five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more. In one embodiment, the invention relates to a formulation comprising self-assembling peptides that undergo sol-gel transition in the presence of an electrolyte solution such as biological fluids and salts. The formulation can provide sustained release of antibody and antibody fragments, in particular, IgG. Antibody diffusivities can be decreased with increasing hydrogel nanofiber density, providing a means to control the release kinetics.

The invention further relates to multilayered hydrogel structures containing substantially concentric spheres of complementary and structurally compatible peptides, for example, ac-$(RADA)_4$-$CONH_2$ core and ac-$(KLDL)_3$-$CONH_2$ shell. The therapeutic agent can be diffused through 'onion-like' architectures that can be formed when complementary peptides are used. In one embodiment, the sustained release formulations of the invention can provide functionally intact antibody release for at least two weeks, three weeks, four weeks, five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more. In a preferred embodiment, the formulation provides sustained release of a functionally intact antibody, for example IgG, for a period of at least about two months to about three months.

In one embodiment, the invention relates to a formulation wherein the secondary and tertiary structure analyses as well as biological assays of the released antibody, for example IgG, where the encapsulation and release did not affect the conformation of the antibody and their biological activity even after two or three months inside the hydrogel. The functionality of polyclonal human IgG can be determined by its affinity to the phosphocholine antigen after IgG encapsulation and release. The binding efficacy to the antigen can be used determine the functional fidelity after encapsulation. The invention relates to a formulation where a fully biocompatible and injectable peptide hydrogel system is used for controlled release applications as a carrier for therapeutic antibodies.

In a preferred embodiment, the invention relates to a sustained release formulation of human immunoglobulin (pI 7.1, MW 146 kDa) comprising -ac-$(RADA)_4$-$CONH_2$ and ac-$(KLDL)_3$-$CONH_2$ self-assembling peptide hydrogels over a period of about 3 months. In a preferred embodiment, the processes involved in incorporating and releasing the antibody from the peptide hydrogels does not substantially affect its conformation and function based on the analysis of the released antibody using circular dichroism (CD), fluorescent spectroscopy and immunoassays. The biological activity of IgG can be monitored using single molecule fluorescence correlation spectroscopy (FCS) and quartz crystal microbalance (QCM) biosensor techniques.

The invention further relates to a sustained drug delivery system that can efficiently direct therapies to specific tissues where the localized delivery results in less toxicity side effects on patients. The injectable self-assembling peptide scaffold system, which gels under physiological conditions, can be used for sustained release applications including immunotherapies to release active antibodies locally in specific tissues over prolonged periods of time. In a preferred embodiment, human antibodies are slowly released through the ac-$(RADA)_4$-$CONH_2$ or ac-$(KLDL)_3$-$CONH_2$ peptide hydrogels for a period of over about 3 months. The release kinetics can be varied with the amino acid sequence of the self-assembling peptides which form the hydrogel and the density of the peptide nanofibers in the hydrogel. Furthermore the controlled release system can present biologically active proteins where secondary and tertiary structure of the hydrogel released antibodies as well as their biological activities were not substantially affected by encapsulation and release through the hydrogel. The programmability of the peptide sequence is unique and allows a means of controlling the nanofiber properties at the molecular level, which, in turn, can alter the biomolecular diffusion and release kinetics.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying drawings in which like reference characters refer to the same parts throughout the different views. The drawings are not necessarily to scale, emphasis instead being placed upon illustrating the principles of the invention.

Figure 1. Graphical representation of (A) the ac-$(RADA)_4$-$CONH_2$ peptide monomer, and of the peptide nanofiber, (B) the IgG molecule, (C) electron microscopy image of the peptide nanofibers, and (D) macroscopic image of the scaffold hydrogel. Color scheme for IgG and peptides: positively charged (blue), negatively charged (red), and hydrophobic (grey).

Figure 2. (A) Schematic representation of the multi-layered hydrogel. (B) Optical and (C-D) fluorescence microscopic examination of the multi-layered hydrogels. For visualization purposes the shell (self-assembled ac-$(KLDL)_3$-$CONH_2$ peptide) is loaded with the Alexa-488 fluorophore (C) and the core (self-assembled ac-$(RADA)_4$-$CONH_2$ peptide) is loaded with CY3 (D).

Figure 3. (A) The release profiles (A) during the entire 3-month period and (B) during the first 12 days for IgG through hydrogels of different peptides and different peptide nanofiber densities. Hydrogels consisted of the self asse mbling peptides (i) ac-$(RADA)_4$-$CONH_2$ with concentration of 0.5 % w/v (light blue, ▲), 1.0 % w/v (blue, ▲), and 1.5 % w/v (dark blue, ▲) and of (ii) ac-$(KLDL)_3$-$CONH_2$ with concentrations 0.3 % w/v (red, ■) and 0.6 % w/v (magenta, ■). Release experiments were performed in PBS, pH 7.4 at room temperature. Data points represent the average of 5 samples with calculated standard deviations less than 12 %. (C) IgG release plotted as a function of the square root of time showing a biphasic diffusion mechanism. The initial linear part of the plots represents simple diffusion of IgG through the peptide hydrogel and is used to calculate diffusion coefficients based on the Fick's law (Equation 1).

Figure 4. The release profiles (A) during the entire 3-month period and (B) during the first 12 days for IgG through hydrogels consisting of ac-$(RADA)_4$-$CONH_2$ with concentration of 1.0 % w/v (blue, ▲), ac-$(KLDL)_3$-$CONH_2$ with concentrations 0.6 % w/v (magenta, ■), and multi-layered, two-component hydrogel which is composed of 1.0 % w/v ac-$(RADA)_4$-$CONH_2$ (core) and 0.6 % w/v ac-$(KLDL)_3$-$CONH_2$ (shell) (yellow, ●). Release experiments were performed in PBS, pH 7.4 at room temperature. Data points represent the average of 5 samples with calculated standard deviations less than 12 %. (C) IgG release plotted as a function of the square root of time showing a biphasic diffusion mechanism. The initial linear part

of the plots represents simple diffusion of IgG through the peptide hydrogel and is used to calculate diffusion coefficients based on the Fick's law (Equation 1).

Figure 5. Spectroscopic examination of the human IgG in PBS, pH 7.4. (A) Far-UV CD spectra of native (solid line) and peptide hydrogel released (broken line) IgG. (B) Normalized fluorescence emission spectra of the native (solid line) and released IgG (broken line) through the peptide hydrogel; excitation wavelength was 300 nm. Spectra were recorded at room temperature in 2-month post release samples.

Figure 6. Translational diffusion times of free IgG molecules in the absence and in the presence of the PC-BSA antigen before and after release through the ac-$(RADA)_4$-$CONH_2$ peptide hydrogel. FCS data showed that the interaction of IgG with the antigen resulted in three different species: free IgG molecules which had diffusion times similar to that of IgG alone, and IgG that bonds to one or two antigen molecules. The relative concentration of these species was similar before and after release of IgG. Standard deviations are between 5 and 12 %.

Figure 7. QCM time-dependent frequency changes, -dF, upon association (binding) and dissociation of the (A) native and (B) hydrogel released polyclonal human IgG to immobilized PC-BSA (antigen). Time zero represents injection of IgG in the flow channel which is followed by the association phase. The biosensor signal (black line) increases upon IgG injection and binding of IgG to the immobilized antigen and decreases upon buffer injection (arrow) which results dissociation of the bound IgG. The similarity of the calculated rate constants and of the affinity constants between native and hydrogel released IgG suggests that the functional characteristics of IgG were not affected upon incorporation and release through the peptide hydrogel. Data fitting is represented by the red line.

Figure 8. Diffusion constants of human immunoglobulin (IgG, MW 146 kDa, hydrodynamic radius r_h = 5.3nm, isoelectric point pI 7.1) in solution using the Stokes-Einstein equation and upon release through the peptide hydrogels using Fick's law. Standard deviations were calculated using error propagation (n=8).

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to a pharmaceutical formulation for sustained delivery of an antibody or an antibody fragment comprising one or more gel forming peptides wherein the formulation exhibits sustained delivery for at least two weeks, three weeks, four weeks, five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more. In one embodiment, the invention relates to a formulation comprising self-assembling peptides that undergo sol-gel transition in the presence of an electrolyte solution such as biological fluids and salts. The formulation can provide sustained release of antibody and antibody fragments, in particular, IgG. Antibody diffusivities can be decreased with increasing hydrogel nanofiber density, providing a means to control the release kinetics.

The invention further relates to multilayered hydrogel structures containing concentric spheres of complementary and structurally compatible peptides, for example, ac-$(RADA)_4$-$CONH_2$ core and ac-$(KLDL)_3$-$CONH_2$ shell. The antibody can be diffused through 'onion-like' architectures that can be formed when complementary peptides are used. The sustained release formulations of the invention can provide functionally intact antibody release for at least two weeks, three weeks, four weeks, five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more. In a preferred embodiment, the formulation provides sustained release of a functionally intact antibody, for example IgG, for a period of at least about two months to about three months.

The invention further relates to a process for preparing a multilayered gel formulation wherein a first core gel comprising a therapeutic agent is formed and one more layers of gels are further formed to encapsulate the core gel. The formation of core gel can be occur upon mixing of the gel forming peptide in a suitable aqueous solution and waiting for the gel to form. In one embodiment, the gel forming peptide is added to a buffer solution and mixed. In one embodiment, a droplet shaped gel can be formed by forming a drop at the tip of a syringe needle and allowing gelation to take place.

In one embodiment, the invention relates to a formulation wherein the secondary and tertiary structure analyses as well as biological assays of the released antibody, for example IgG, where the encapsulation and release did not affect the conformation of the antibody and their biological activity even after two or three months inside the hydrogel. The functionality of polyclonal human IgG can be determined by its affinity to the phosphocholine antigen after IgG encapsulation and release. The binding efficacy to the antigen can be used

determine the functional fidelity after encapsulation. The invention relates to a formulation where a fully biocompatible and injectable peptide hydrogel system is used for controlled release applications as a carrier for therapeutic antibodies.

The release kinetics for human immunoglobulin (IgG) through the permeable structure of nanofiber scaffold hydrogels consisting of the ac-$(RADA)_4$-$CONH_2$ and ac-$(KLDL)_3$-$CONH_2$ self-assembling peptides were studied during a period of 3 months. Self-assembling peptides are a class of stimuli-responsive materials, which undergo sol-gel transition in the presence of an electrolyte solution such as biological fluids and salts. IgG diffusivities decreased with increasing hydrogel nanofiber density, providing a means to control the release kinetics. Multilayered hydrogel structures were also created consisting of concentric spheres of ac-$(RADA)_4$-$CONH_2$ core and ac-$(KLDL)_3$-$CONH_2$ shell. The antibody diffusion profiles were determined through the 'onion-like' architectures. Secondary and tertiary structure analyses as well as biological assays of the released IgG showed that encapsulation and release did not affect the conformation of the antibody and their biological activity even after 3 months inside the hydrogel. The functionality of polyclonal human IgG to the phosphocholine antigen was determined and showed that IgG encapsulation and release did not affect the antibody binding efficacy to the antigen. Our results show that this fully biocompatible and injectable peptide hydrogel system may be used for controlled release applications as a carrier for therapeutic antibodies.

The invention further relates to a sustained drug delivery system that can efficiently directs therapies to specific tissues where the localized delivery results in less toxicity side effects on patients. The injectable self-assembling peptide scaffold system, which gels under physiological conditions, can be used for sustained release applications including immunotherapies to release active antibodies locally in specific tissues over prolonged periods of time. In a preferred embodiment, human antibodies are slowly released through the ac-$(RADA)_4$-$CONH_2$ or ac-$(KLDL)_3$-$CONH_2$ peptide hydrogels for a period of over about 3 months. The release kinetics can be varied with the amino acid sequence of the self-assembling peptides which form the hydrogel and the density of the peptide nanofibers in the hydrogel. Furthermore the controlled release system can present biologically active proteins where secondary and tertiary structure of the hydrogel released antibodies as well as their biological activities were not substantially affected by encapsulation and release through the hydrogel. The programmability of the peptide sequence is unique and allows a means of

controlling the nanofiber properties at the molecular level, which, in turn, can alter the biomolecular diffusion and release kinetics.

In one embodiment, the invention relates to core comprising a first peptide monomer and an antibody wherein said core is coated with a layer forming second peptide. In a preferred embodiment, the core comprises a first peptide selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$; and a said peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$, Ac-$(KLDL)_5$-$CONH_2$; and said antibody is an IgG antibody. The multi-layered hydrogel structures can be prepared in a two-step gelation process. For example, upon mixing the ac-$(RADA)_4$-$CONH_2$ peptide solution (about 0.2 to about 5.0% w/v; preferably between about 0.4 to about 2.0% w/v; preferably between about 0.5 to about 1.5% w/v) with a buffer solution (PBS or another buffer suitable for gel formation) containing IgG (about 1μM to about 20μM; preferably between about 2μM to about 10μM; 4μM to about 8μM) a gel containing IgG can be formed. The gel can be formed into a desirable physical shape. For example, using a syringe to form a drop at the tip of needle and waiting for sufficient time to allow for gelation can result in substantially spherical core structure. The self-assembly process can result in a hydrogel with substantially spherical geometry hanging at the tip of the syringe needle. The first gel formed can be coated with the second layer of self-assembling peptide to give a layered product. For example, using a second syringe, a layer of another self-assembling peptide, ac-$(KLDL)_3$-$CONH_2$ can be applied on the outer surface of the core. The ac-$(KLDL)_3$-$CONH_2$ peptide solution can be about 0.1 to about 5.0% w/v; preferably between about 0.2 to about 2.0% w/v; preferably between about 0.3 to about 1.5% w/v; or more preferably about 0.6%w/v. In a final step, before gelation occurred allowing a drop of the ac-$(KLDL)_3$-$CONH_2$ peptide solution to come in contact and encapsulate the preformed ac-$(RADA)_4$-$CONH_2$ peptide hydrogel can result in an 'onion-like' multi-layered hydrogel structure. The above steps of forming the core with IgG antibody and a self-assembling peptide, followed by coating with a second peptide can be repeated multiple times to result in an multilayered onion-like structure. For example, the above described product containing the ac-$(RADA)_4CONH_2$/IgG core coated with ac-$(KLDL)_3$-$CONH_2$ can be further coated with a layer of ac-$(RADA)_4CONH_2$/IgG followed by another coating of ac-$(KLDL)_3$-$CONH_2$ based on the desired release profile.

Other self-assembling peptides can also be used for forming one or more layers or the core of the formulation. Examples of such peptides include: K27, a twenty seven amino acid beta sheeting forming peptide (KLEALYIIMVLGFFGFFILGIMLSYIR); K24, a twenty four amino acid beta sheet forming peptide (KLEALYVLGFFGFFILGIMLSYIR); or SER-1 described in Boden et al. (U.S. Patent Publication 20020132974); or $PF4_{24-46}$ described in Lockwood et al. (Biomacromolecules, 2002, 3 (6), pp 1225–1232).

Other gel forming or coating agents such as hyaluronic acid, collagen, crosslinked polymers, glycosaminoglycans, keratin, fibronectin, vitronectin, or laminin, can be used to form one or more layers encapsulating the core of the formulation described herein.

The invention relates to formulations containing one or more therapeutic agents. In a preferred embodiment, the therapeutic agent is selected from an antibody, an IgG antibody, a monoclonal antibody, a polyclonal antibody, a monoclonal antibody fragment and a polyclonal fragment, wherein the therapeutic agent is release over a period of at least about one month, or two months or three months.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g. bispecific antibodies), and antibody fragments so long as they exhibit the desired biological activity.

"Antibody fragments" comprise a portion of a full length antibody, generally the antigen binding or variable region thereof. Examples of antibody fragments include Fab, Fab', F(ab').sub.2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules; and multispecific antibodies formed from antibody fragments.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. In contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determination on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies

to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., Nature 256:495 (1975), or may be made by recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the technique described in Clackson et al., Nature 352:624-626 (1991) and Marks et al., J. Mol. Biol. 222:581-597 (1991), for example.

In a preferred embodiment, an antibody is selected from IgD, IgA, IgM, IgE, or IgG immunoglobulin. In a more preferred embodiment, the antibody is IgG. In a preferred embodiment, the antibody is selected from the group consisting of: IgG1, IgG2, IgG3 and IgG4, IgM1 and IgM2, and IgA1 and IgA2 antibodies. In one embodiment, the antibody is whole antibody or single-chain Fv antibody fragment or Fab antibody fragment. In a preferred embodiment, the antibody is selected from Rituximab, Infliximab, Trastuzumab, Abciximab, Palivizumab, Murumonab-CD3, Gemtuzumab, Trastuzumab, Basiliximab, Daclizumab, Etanercept, and Ibritumomab tiuxetan. In one embodiment, the antibody is selected from anti-TNF antibodies, anti-CD3 antibodies, anti-CD20 antibodies, anti-CD25 antibodies, anti-CD33 antibodies, anti-CD40 antibodies anti-HER2 antibodies, anti-HBV antibodies, anti-HAV antibodies, anti-HCV antibodies, anti-GPIIb/IIIa receptor antibodies, anti-RSV antibodies, anti-HIV antibodies, anti-HSV antibodies and anti-EBV antibodies.

Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit or nonhuman primate having the desired specificity, affinity, and capacity. In some instances, FR residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues which are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones

et al., Nature 321:522-525 (1986); Riechmann et al., Nature 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol. 2:593-596 (1992).

"Single-chain Fv" or "sFv" antibody fragments comprise the V_H and V_L domain of antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the sFv to form the desired structure for antigen binding. For a view of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds. Springer-Verlag, N.Y., pp. 269-315 (1994).

IgG is the most abundant class of antibody in the blood (serum concentration is 13 mg/ml). There are four subclasses of IgG which are all monomeric and they usually have a very high affinity for antigen. The subclasses of antibody IgG produced is dependant on the cytokines present (especially IL-4 and IL-2) and each class has its own special activity. IgG also plays an important role in neutralizing toxins (from bacterial infection for example) in the blood and tissues.

An antibody "retains its biological activity" in a pharmaceutical formulation, if the change in biological activity of the antibody at a given time is within about 20% (within the errors of the assay) of the biological activity exhibited at the time the pharmaceutical formulation was prepared as determined in an antigen binding assay, for example.

A variety of therapeutic agents such as proteins, polypeptides, micro-RNA and other biopolymers can also be incorporated into the formulation for sustained release. In one embodiment, the therapeutic agent is selected from erythropoietin (EPO), interferon-alpha, interferon-beta, interferon-gamma, growth hormone, growth hormone releasing factor, nerve growth factor (NGF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), blood clotting factor, insulin, oxytocin, vasopressin, adrenocorticotropic hormone, epidermal growth factor, platelet-derived growth factor (PDGF), prolactin, luliberin, luteinizing hormone releasing hormone (LHRH), LHRH agonists, LHRH antagonists, somatostatin, glucagon, interleukin-2 (IL-2), interleukin-11 (IL-11), gastrin, tetragastrin, pentagastrin, urogastrone, secretin, calcitonin, enkephalins, endorphins, angiotensins, thyrotropin releasing hormone (TRH), tumor necrosis factor (TNF), tumor necrosis factor related apoptosis inducing ligand (TRAIL), heparinase, bone morphogenic protein (BMP), human atrial natriuretic peptide (hANP), glucagon-like peptide (GLP-1), renin, bradykinin,

bacitracins, polymyxins, colistins, tyrocidine, gramicidins, cyclosporins, small interference RNA (siRNA), plasmid DNA, and antisense oligodeoxynucleotide (AS-ODN).

The therapeutic agents used as anti-acne agents, anti-allergic agents, anxiolytics, anti-asthmatics, anticancer agents, hypolipemic agents, hormonal contraceptives, antidepressants, antidiabetic agents, antalgics, antiasthenics, antihypertensives, antifungals, antibiotics, sleeping drugs, hormone treatments, antimigraine agents, drugs used to treat excess weight, antiparkinsonian agents, neuroleptics, nonsteroidal anti-inflammatories, ovulation inducers, mucolytics, antitussives, erection inducers and antiulceratives can be incorporated into the core structure of the formulations herein for sustained delivery. In a preferred embodiment, the invention relates to formulations comprising a therapeutic agent selected from insulin, leuprolide, gonadorelin, somatostatin, octreotide, enfuviritide, calcitonin and nesiritide.

In another embodiment, the therapeutic agent is a thrombolytic agent selected from among ancrod, anistreplase, bisobrin lactate, brinolase, Hageman factor (i.e., factor XII) fragments, molsidomine, plasminogen activators such as streptokinase, tissue plasminogen activators (TPA), urokinase, TFPI, plasmin and plasminogen and inhibitors of coagulation factors IIa, Va, VIIa, VIIIa, IXa, Xa, XIa, XIIa and XIIIa.

In another embodiment, the therapeutic agent is a cell signaling molecule selected from among a cytokine, a growth factor, an interleukin, α-interferon, γ-interferon, transforming growth factor-β, neutrophil activating peptide-2 and its analogs, macrophage inflammatory protein and its analogs, and platelet-factor 4 and transforming growth factor-beta, and combinations thereof.

In another embodiment, the therapeutic agent is an anti-inflammatory agent selected from among alclofenac, alclometasone dipropionate, algestone acetonide, alpha amylase, amcinafal, amcinafide, amfenac sodium, amiprilose hydrochloride, anakinra, anirolac, anitrazafen, apazone, aspirin, balsalazide disodium, bendazac, benoxaprofen, benzydamine hydrochloride, bromelains, broperamole, budesonide, carprofen, cicloprofen, cintazone, cliprofen, clobetasol propionate, clobetasone butyrate, clopirac, cloticasone propionate, cormethasone acetate, cortodoxone, deflazacort, desonide, desoximetasone, dexamethasone dipropionate, diclofenac potassium, diclofenac sodium, diflorasone diacetate, diflumidone sodium, diflunisal, difluprednate, diftalone, dimethyl sulfoxide, drocinonide, endrysone, enlimomab, enolicam sodium, epirizole, etodolac, etofenamate, felbinac, fenamole, fenbufen, fenclofenac, fenclorac, fendosal, fenpipalone, fentiazac, flazalone, fluazacort, flufenamic

acid, flumizole, flunisolide acetate, flunixin, flunixin meglumine, fluocortin butyl, fluorometholone acetate, fluquazone, flurbiprofen, fluretofen, fluticasone propionate, furaprofen, furobufen, halcinonide, halobetasol propionate, halopredone acetate, ibufenac, ibuprofen, ibuprofen aluminum, ibuprofen piconol, ilonidap, indomethacin, indomethacin sodium, indoprofen, indoxole, intrazole, isoflupredone acetate, isoxepac, isoxicam, ketoprofen, lofemizole hydrochloride, lomoxicam, loteprednol etabonate, meclofenamate-sodium, meclofenamic acid, meclorisone dibutyrate, mefenamic acid, mesalamine, meseclazone, methyl-prednisolone suleptanate, morniflumate, nabumetone, naproxen, naproxen sodium, naproxol, nimazone, olsalazine sodium, orgotein, orpanoxin, oxaprozin, oxyphenbutazone, paranyline hydrochloride, pentosan polysulfate sodium, phenbutazone sodium glycerate, pirfenidone, piroxicam, piroxicam cinnamate, piroxicam olamine, pirprofen, prednazate, prifelone, prodolic acid, proquazone, proxazole, proxazole citrate, rimexolone, romazarit, salcolex, salnacedin, salsalate, salicylates, sanguinarium chloride, seclazone, sermetacin, sudoxicam, sulindac, suprofen, talmetacin, talniflumate, talosalate, tebufelone, tenidap, tenidap sodium, tenoxicam, tesicam, tesimide, tetrydamine, tiopinac, tixocortol pivalate, tolmetin, tolmetin sodium, triclonide, triflumidate, zidometacin, glucocorticoids and zomepirac sodium.

In another embodiment, the therapeutic agent is an ADP receptor antagonist selected from among clopidogrel, ticlopidine, prasugrel, sulfinpyrazone, AZD6140, AZD6933 and AR-C69931; a glycoprotein IIb/IIIa receptor inhibitor selected from among abciximab, fradafiban, lamifiban, lotrafiban, orbofiban, roxifiban, sibrafiban, tirofiban and xemilofiban.

In another embodiment, the therapeutic agent is an anti-coagulant agent selected from among a vitamin K antagonist, coumarin and coumarin derivatives, warfarin sodium; a heparin; ardeparin sodium; bivalirudin; bromindione; coumarin dalteparin sodium; desirudin; dicumarol; lyapolate sodium; nafamostat mesylate; phenprocoumon sulfatide; and tinzaparin sodium.

In another embodiment, the therapeutic agent is a lipid reducing agent selected from among bezafibrate, ciprofibrate, clofibrate, etofibrate, fenofibrate, gemfibrozil, ronifibrate and simfibrate; a statin, atorvastatin, cerivastatin, fluvastatin, lovastatin, mevastatin, pitavastatin, pravastatin, rosuvastatin and simvastatin; niacin or a niacin derivative, including acipimox, aluminium nicotinate, niceritrol (penta-erythritol tetranicotinate), nicofuranose and nicotinyl alcohol; a bile acid sequestrant, including colesevelam, colestyramine, colestipol

and colextran; a CETP inhibitor, such as anacetrapib; benfluorex; cholestyramine; dextrothyroxine; ezetimibe; laropiprant; meglutol; omega-3-triglycerides; policosanol; probucol; and tiadenol.

In another embodiment, the therapeutic agent is a cyclooxygenase-2 (COX-2) inhibitor selected from among aspirin, celecoxib, lumiracoxib and etoricoxib.

In another embodiment, the therapeutic agent is a an angiotensin system inhibitor selected from among an angiotensin converting enzyme (ACE) inhibitor, an angiotensin II receptor antagonist, an agent that activates the catabolism of angiotensin II, and an agent that prevents the synthesis of angiotensin I from which angiotensin II is ultimately derived; an ACE inhibitor selected from among benazepril, captopril, cilazapril, delapril, enalapril, fosinopril, imidapril, lisinopril, moexipril, perindopril, quinapril, ramipril, spirapril, temocapril, trandolapril and zofenopril; an angiotensin II receptor antagonist selected from among azilsartan, candesartan, eprosartan, irbesartan, losartan, olmesartan, tasosartan, telmisartan and valsartan; a chemotherapeutic anti-cancer drug selected from among busulfan, carmustine, chlorambucil, cyclophosphamide, doxorubicin, estramustine, hepsulfan, hydroxy-carbamide, ifosfamide, lomustine, melphalan, methotrexate, pipobroman and thioTEPA.

In another embodiment, the therapeutic agent is a thromboxane synthetase inhibitor selected from among aspirin, .beta.-[4-(2-carboxy-1-propenyl)benzyl]pyridine hydrochloride (OKY-1555) and 1-carboxyhexyl-, 1-carboxyheptyl, and 1-carboxy-octyl-imidazoles, 4(Z)-6-[(4RS,5SR)-4-(2-hydroxyphenyl)-1,3-dioxan-5-yl]hex-4-enoic acid, BM-573, camonagrel, CGS-12970, daltroban, dazmegrel, DTTX30, E-6700, FCE-27262, imitrodast (CS-518), isbogrel (CV-4151), ketoconazole, KK-505, KY-063, nafagrel (DP-1904), ozagrel (OKY-046), picotamide, pirmagrel (CGS-13080), ridogrel, SQ29548, rolafagrel (FCE-22178), satigrel (E-5510), sulotroban, terbogrel and UK 38485.

In another embodiment, the therapeutic agent is a JAK-2 inhibitor selected from among AT9283, VX-680, MK0457, TG101209, INCB018424, LS104, XLO19, TG101348, vorinostat, a 4-aryl-2-amino-pyridine and a 4-aryl-2-aminoalkyl-pyridine.

A "therapeutically effective amount" of a therapeutic agent refers to an amount effective in the prevention or treatment of a disease or disorder for the treatment of which the antibody is effective. A "disease or disorder" is any condition that would benefit from

treatment with the antibody. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question.

The peptide sequences are presented as capped at the amino terminus with acetyl group. However, the peptides without amino terminus capping can also be used. The peptide sequences are synthesized with a carbamoyl group at the C-terminus. However, they can also be used with a carboxylic acid C-terminus.

EXAMPLES

MATERIALS AND METHODS

Chemicals and reagents. The ac-$(RADA)_4$-$CONH_2$ and ac-$(KLDL)_3$-$CONH_2$ peptides were obtained in powder form CPC Scientific (Sunnyvale, CA, USA) and were characterized at the MIT Biopolymers Lab (Cambridge, MA, USA). Human polyclonal IgG was purchased from Sigma-Aldrich (St. Louis, MO, USA). The pI 7.1 of human IgG was determined by isoelectric focusing gel electrophoresis in a PhastSystem, using IPG strips and protein standards (Bio-Rad Laboratories, Hercules, CA) and the IPGphor system (Amersham Pharmacia Biotech, Uppsala, Sweden).

IgG release experiments. Peptide hydrogels were formed using well-established protocols [9,10,11,12,13]. Briefly, the ac-$(RADA)_4$-$CONH_2$ and ac-$(KLDL)_3$-$CONH_2$ peptides were dissolved in deionized water and ultra-sonicated using a probe sonicator for 30 min prior to use. The peptide solutions in water was mixed with phosphate buffered saline (PBS, pH=7.4) containing IgG at a final concentration of 5μM. 40μl of the mixture was transferred into 200μl polypropylene tubes and gelation occurred ~10 min. Subsequently, 70μl of PBS was slowly added to the 40μl of the hydrogel. To satisfy the perfect-sink conditions and allow for the determination of the protein release profile, 40μl of the supernatant was replaced with the same volume of fresh PBS at frequent time points. During the course of the measurements the hydrogel volume did not change and therefore, IgG release could not be attributed to hydrogel degradation or swelling. Experiments showed the absence of any detectable adsorption of IgG on the surface of the polypropylene tubes.

The formation of the multi-layered hydrogel structures involved a two-step gelation process. Upon mixing the ac-$(RADA)_4$-$CONH_2$ peptide solution (1.0% w/v) with the PBS solution which contained 5μM IgG, we used a syringe to form a drop at the tip of needle and waited 10 min to allow for gelation. The self-assembly process resulted in a hydrogel with

spherical geometry hanging at the tip of the syringe needle. Then, using a different syringe, we mixed the ac-$(KLDL)_3$-$CONH_2$ peptide solution in water with PBS to have a solution with final peptide concentration of 0.6% w/v. In a final step, before gelation occurred we carefully allowed a drop of the ac-$(KLDL)_3$-$CONH_2$ peptide solution to come in contact and encapsulate the preformed ac-$(RADA)_4$-$CONH_2$ peptide hydrogel thus creating an 'onion-like' multi-layered hydrogel structure (Figure 2). The multi-layered hydrogel was placed in the bottom of a polypropylene tube and diffusion experiments were performed as described in the previous paragraph (Figure 2A).

The IgG release experiments through the self-assembling peptide nanofiber scaffold hydrogel were performed at room temperature. The concentration of the released IgG in the supernatant was determined spectrophotometrically at 280nm (NanoDrop ND-1000 UV-Vis, NanoDrop Technologies, Delaware, USA). All data points represent the average of 4 samples. Uncertainties in the calculated parameters were estimated via common error propagation techniques, i.e., for a function $y = g(x_1, x_2)$ errors in the calculated values were determined using the equation $\sigma_y^2 = (\partial g/\partial x_1)^2 \sigma_{x_1}^2 + (\partial g/\partial x_2)^2 \sigma_{x_2}^2$.

Microscopic visualization of the multilayered, two-component hydrogel system. Prior to visualization of the multi-layered hydrogel we mixed the ac-$(KLDL)_3$-$CONH_2$ and the ac-$(RADA)_4$-$CONH_2$ peptide solutions with the Alexa-488 (green) and CY3 (red) fluorescent dyes solutions, respectively. The multi-layered hydrogel consists of two concentric spheres with composition 1.0% w/v ac-$(RADA)_4$-$CONH_2$ (core) and 0.6% w/v ac-$(KLDL)_3$-$CONH_2$ (shell). Visualization was performed with a fluorescence microscope (Nikon TE300 equipped with a Hamamatsu camera and Openlab image acquisition software).

Diffusivity determination from released IgG concentration. For a hydrogel matrix that contains a molecularly dispersed diffusing agent, the apparent diffusion coefficient may be calculated using the 1-D unsteady-state form of Fick's second law of diffusion, which for small values of time (t) is given by [21,22]:

$$\frac{M_t}{M_\infty} = \left(\frac{16\, D_{\text{app.}}\, t}{\pi\, H^2} \right)^{0.5} \qquad (1),$$

where $D_{app.}$ is the apparent diffusivity, M_t and M_∞ are the cumulative mass of the diffusing compound released from the hydrogel after t and infinite time (∞), respectively. The thickness of the hydrogel matrix (H) inside the well was calculated from the volume of the

peptide-IgG solution (i.e., 40 µl) and from the dimensions of the wells of the polypropylene tubes. $D_{app.}$ may be obtained from the slope of the straight line fitting the data of M_t/M_∞ vs. $t^{0.5}$, for $0 < M_t/M_\infty < 0.6$.

Circular Dichroism (CD). Far-UV CD spectra were recorded between 190nm – 260nm at room temperature (Aviv 62DS spectrometer). CD spectra of hydrogel released IgG after 2-month were compared with those of freshly prepared IgG solutions at the same concentration. Spectra were recorded in 1nm steps and averaged over 2 seconds. All measurements were carried out in 1-mm quartz cuvettes in PBS, pH7.4. Spectra resulted from accumulation and averaging of 4 scans. Blank spectra of the buffer without IgG, obtained under identical conditions, were subtracted.

Fluorescence emission spectroscopy. Fluorescence emission of released and native IgG was measured using a Perkin-Elmer LS-50B spectrophotometer at room temperature using quartz cuvettes of 1cm path length. Emission spectra were recorded between 310nm – 400nm on excitation at 300nm. The excitation and emission slit widths were set at 5.0nm and 2.5nm, respectively. Sample conditions were identical to those described for the CD measurements.

FCS functionality assay of IgG. FCS characterization of the antibody-antigen binding was performed at room temperature using the Fluoropoint single molecule detection system (Olympus, Tokyo, Japan). The fluorescence signal of proteins is small for detection by the single molecule analysis system that we used and therefore, we labeled human IgG with the strong fluorophore Alexa-647 using a standard protein labeling kit (Molecular Probes Inc., Eugene, OR). Alexa-647 fluorescently labeled IgG molecules crossing the confocal femtoliter volume were excited with a He-Ne laser at 633nm, were measured (counts per unit time) and converted to concentration using a calibration curve of the respective fluorescently labeled IgG at different concentrations.

Fluctuations in fluorescent intensity within a confocal volume are recorded as a function of time and the autocorrelation function, $g(\tau)$, is influenced by the properties of the fluorescing molecules as well as the diffusion dynamics in the local environment [16,17]. To

16 R. Rigler, E.S. Elson, Fluorescence correlation spectroscopy: Theory and applications, Springer, NY, 2001.

17 W.W. Webb, Fluorescence correlation spectroscopy: inception, biophysical experimentations, and prospectus, Appl. Opt. 40 (2001) 3969–3983.

define the autocorrelation function for the case of anomalous 3-D diffusion of monodisperse particles in solution [16,[18],[19]], the Fluoropoint system employed Equation 2:

$$g(\tau)=1+\left(\frac{1-F_{trip}+F_{trip}e^{-\tau/\tau_{trip}}}{N}\right)\left(\frac{1}{1+(\tau/\tau_D)}\right)\left(\frac{1}{1+(1/s^2)(\tau/\tau_D)}\right)^{0.5} \quad (2),$$

where $g(\tau)$ is a function of the fractional population (F_{trip}) and decay time (τ_{trip}) of the triplet state, N is the number of molecules within the sample volume, τ_D is the translational diffusion time, and s is a factor describing the cylindrically shaped detection volume and is equal to the ratio of the radius of the cylinder's basal plane (ω_o) divided by one half of its height (ω_1). In a fully anisotropic solution, with diffusing molecules significantly smaller than the confocal volume, the diffusion coefficient D of the molecules (e.g., IgG) is equal to $D=\omega_o^2/4\tau_D$. The autocorrelation profile was fitted using single and multiple translational diffusion times. All data points represent the average of 4 or 8 samples.

Alexa-647 labeled IgG in PBS was allowed to interact for 1 hour at room temperature with 10-fold excess PC-BSA antigen (Athera Biotechnologies AB, Sweden). Any interaction between IgG and antigen would result in increased molecular weight (and size) complexes, which would be characterized by slower translational diffusion times (τ_D) compared to free IgG. The effect of non-specific binding of IgG to the antigen was evaluated by measuring the binding affinity in the presence of different concentrations of Tween detergent; at 0.5% Tween non-specific binding was minimal and therefore all IgG-antigen binding studies were performed at this Tween concentration. The data were analyzed using FCS algorithms as described above and the autocorrelation functions were fitted using one and multiple components.

QCM functionality assay of IgG. Biological activity of native and hydrogel released human IgG was also assessed by QCM (Attana A200, Stockholm, Sweden). The setup consists of a thin piezoelectric quartz disk having electrodes on each side. When connected to an oscillating current, the quartz crystal oscillates at resonant frequencies, which are sensitive to the crystal mass; as material adsorbs to the surface of the crystal the recorded frequency

18 M.J. Saxton, Anomalous diffusion due to obstacles: a Monte Carlo study, Biophys. J. 66 (1994) 394-401.

19 O. Krichevsky, G. Bonnet, Fluorescence correlation spectroscopy: the technique and its applications, Rep. Prog. Phys. 65 (2002) 251-297.

changes. To compare the binding properties of the native and hydrogel released human IgG, the PC-BSA antigen was covalently immobilized to channels A and B using an amine coupling kit. Preliminary experiments showed that non-specific binding was minimal in 0.05% Tween detergent and therefore, all IgG-antigen binding studies were performed at this Tween concentration.

Kinetic data at room temperature were obtained by injecting 35μL of 20 μg/mL human IgG for 84 seconds at 20μL/min flow rate over PC-BSA immobilized on the QCM surface and measuring the mass increase of the surface due to IgG binding to PC-BSA. The kinetic data were corrected for negative controls, which consist of the signals observed upon injection of; (i) human polyclonal IgG over a surface with immobilized BSA, (ii) monoclonal anti-His tag antibody over a surface with immobilized PC-BSA, and (iii) anti-rhodopsin antibody over a surface with immobilized PC-BSA. The data were analyzed using the Attaché Evaluation software using a simple 1:1 binding model for the binding reaction event between the antibody and the antigen. From the analysis the association, k_a, and dissociation, k_d, rate constants were calculated as well as the binding affinity constants, defined as $K_D = k_d / k_a$, of the native and released IgG for the antigen. The fitting was evaluated based on the χ^2 value and the residual error which was near zero.

RESULTS AND DISCUSSION

Human IgG release through the peptide scaffold hydrogel. As seen in Figure 3A, an initial rapid release of IgG was observed within the first hour (burst effect). This is likely due to IgG molecules that were at or near the solvent/hydrogel interface and rapidly escaped into the supernatant solution. Previous work showed that protein release through this peptide hydrogel depends on the size of the protein [10]. Small proteins are released faster whereas IgG, which is a large protein (~150kD), was released slower. Therein, it was shown that IgG release was not complete after 3 days. Here we show that IgG release through peptide scaffold hydrogels did not asymptotically reach a plateau value even after 3 months. In hydrogel systems protein release rarely reaches 100%, the reason being the physical entrapment of the protein molecules in highly entangled nanofiber domains of the hydrogel, which do not allow free motion of the diffusant. However, self-assembling peptide hydrogels are biodegradable and therefore, upon introduction into a living organism the hydrogels will be decomposed to single peptide monomers and subsequently to amino acids. This process

will allow for the entire content load in the scaffold hydrogel to be released into the host tissue.

The effect of peptide scaffold hydrogel density on the diffusion of human IgG. To investigate the effect of the hydrogel density on the release profiles of IgG, the self-assembling peptide concentration was varied. Increasing the peptide concentration resulted in higher density network of nanofibers, which hindered the release of IgG. Figure 3A shows the release kinetics of IgG through hydrogels with ac-$(RADA)_4$-$CONH_2$ peptide hydrogels with peptide concentrations of 0.5% w/v (99.5% water), 1.0% w/v (99% water), and 1.5% w/v (98.5% water) and though ac-$(KLDL)_3$-$CONH_2$ peptide hydrogels with concentrations of 0.3% w/v (99.7% water) and 0.6% w/v (99.4% water). Higher density ac-$(KLDL)_3$-$CONH_2$ hydrogels were not tested because such hydrogel are characterized by increased stiffness which made sample handling difficult. The results show that IgG release through the hydrogel may be controlled by varying the peptide nanofiber density.

Building complex multi-layered peptide hydrogel structures for drug release. The two-layer hydrogel system consists of two compartments. Figures 2A and 2B-D show the structure of the two-component peptide hydrogel in which each of the two components is loaded with a dye to facilitate visualization. The hydrogel sphere in the core is formed by self-assembly of the ac-$(RADA)_4$-$CONH_2$ peptide and contains the CY3 dye (red) whereas the encapsulating second sphere is composed by ac-$(KLDL)_3$-$CONH_2$ and contains the Alexa-488 (green) fluorophore. Bright-field and fluorescence microscopy show the contour of each hydrogel (Figure 2B-D). Although diffusion of the dyes eventually results in diffusion of CY3 and Alexa-488 into the ac-$(KLDL)_3$-$CONH_2$ and the ac-$(RADA)_4$-$CONH_2$ hydrogels, respectively and homogeneous distribution throughout the entire two-component peptide hydrogel system is expected, the initial distribution of the dyes clearly defines the shape and dimensions of each hydrogel sphere.

In the antibody release experiments, the core which is formed by gelation of 1.0% ac-$(RADA)_4$-$CONH_2$ peptide is loaded with the antibody whereas the second layer (shell) consists of the ac-$(KLDL)_3$-$CONH_2$ hydrogel does not contain antibody and it encapsulates the core. The formation of the multilayered hydrogel resulted in a system in which the initial protein burst release was significantly smaller compared to that observed in the one-component hydrogels (Figure 3A and 4A). Therefore, IgG diffusion through a multilayered hydrogel results to an apparent near-zero-order diffusion profile (Figure 4A & 4B).

The multilayer self-assembling peptide hydrogel technology is easily transferable from bench to bedside by injecting the two peptide solutions simultaneously using for instance a two-compartment syringe with concentric needles. Gelation occurs upon interaction of the peptide solutions with biological fluids and the release of the active compound could be continuous for prolonged periods of time.

IgG diffusivity through the hydrogel scaffold. The diffusion coefficient of IgG in water at infinitely dilute conditions at 20°C was calculated to be 0.4×10^{-10} m^2 s^{-1} using the Stokes-Einstein equation $D_{S-E} = k_B T / 6\pi\eta r_h$, where k_B is the Boltzmann constant, T is the absolute temperature of the medium, η is the dynamic viscosity of the solvent (taken as 1.002 cP) and r_h is the hydrodynamic radius of IgG [20]. It should be noted however, that when micromolar IgG concentration is used, as in the present work, single molecule analysis showed that the Stokes-Einstein equation overestimates the IgG diffusivities in solution by 10-20% [10]. The Stokes-Einstein equation was developed for infinitely diluted molecules, which follow a Brownian motion and therefore, molecular crowding at micromolar concentrations may affect diffusion by slowing the molecular motion.

To calculate the diffusion coefficient of IgG during release through the peptide hydrogels we used the release profiles shown in Figure 3. Application of the commonly used Fickian model, which is described by Equation 1, results in apparent IgG diffusion coefficients inside the hydrogel which significantly differed (i.e., between 50% and 80%) from those determined for IgG in solution using the Stokes-Einstein equation. The model of calculating diffusion coefficients using Fick's Law was developed assuming diffusion of small molecules, infinite dilution of the diffusant, and that the diffusion of the molecules through the hydrogel solely depends on Brownian motion. Equation 1 is commonly used to determine apparent diffusion coefficients even when these conditions do not apply. The reason for doing so is that it facilitates the discussion of systems when there is no other easily transferable method for determining the diffusivity. In hydrogel systems these assumptions are rarely satisfied. Plotting the release data as a function of the square root of time (Figure 3C) showed that the diffusion mechanism is biphasic. The initial linear part of each plot indicates diffusion controlled release of the IgG through the peptide hydrogel and it is used to

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calculate diffusion coefficients based on Fick's law (Equation 1) [21,22]. Deviation from the straight line at longer times may be associated with non-Fickian, anomalous diffusion. Hydrogel pores with small sizes and/or diffusion hindrance due to specific interactions between diffusing IgG molecules and peptide nanofibers of the hydrogel may also account for the deviation from Fick's law.

Conformational properties of released IgG. Protein aggregation events as well as protein-peptide interactions resulting in protein inactivation could occur during IgG's residence in the peptide solution, during self-assembly and nanofiber formation, or during the release process. To obtain insight into the conformational state of the IgG released from the peptide hydrogel 2-month post encapsulation far-UV CD and fluorescence spectroscopy were employed to examine the secondary and tertiary structural characteristics, respectively.

CD spectra of the native IgG were identical to those reported in the literature for IgG [23]. As may be seen in Figure 5A, the CD spectrum of hydrogel released IgG closely resembled that of a freshly prepared IgG solution. Small deviations were observed in some spectra at wavelengths below 200 nm where the diode or HT (i.e., total absorption) level was high. The relatively low concentration of released IgG, in conjunction with the possible presence of detached nanofibers from the scaffold could be reasons for the low signal-to-noise ratio observed in this region of the spectrum. However, the β-sheet content of IgG, as seen from the 218nm ellipticity, was not affected during the release process.

Fluorescence emission spectra were recorded with excitation at 300nm to excite the tryptophans. The emission is sensitive to the tryptophan microenvironment within the 3D structure of the protein and therefore, fluorescence emission can be used to detect tertiary structure changes of IgG released through the hydrogel. A red shift in the wavelength of maximum emission would indicate protein unfolding as a result of tryptophan exposure to the polar solvent. The fluorescence emission spectrum of IgG was similar with literature reports [24]. Inspection of Figure 5B shows that at the same IgG concentration the emission spectrum

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22 T. Higuchi, Mechanism of sustained-action medication. Theoretical analysis of rate of release of solid drugs dispersed in solid matrices, J. Pharm. Sci. 52 (1963) 1145-1149.

23 K. Ikeda, K. Hamaguchi, S. Migita, Circular dichroism of Bence-Jones proteins and immunoglobulins G, J. Biochem. 63 (1968) 654-660.

24 Y. Nishiyama, Y. Mitsuda, H. Taguchi, S. Planque, M. Hara, S. Karle, C.V. Hanson, T. Uda, S. Paul, Broadly distributed nucleophilic reactivity of proteins coordinated with specific ligand binding activity, J Mol. Recognit. 18 (2005) 295–306.

of released IgG was similar to that of native IgG with respect to both the emission maximum and fluorescence intensity suggesting that IgG encapsulation and release did not induce tertiary structure changes.

Functionality assays of released human IgG. As a subpopulation of human IgGs is known to bind phosphorylcholine (PC), a common antigen which is present in many human infecting microorganisms including *Streptococcus pneumoniae* [25,26,27,28], the PC-conjugated BSA (PC-BSA) antigen was used to examine the functionality of native and hydrogel-released IgG. The PC-BSA sample that was used in this work contains approximately 17 PC molecules per BSA. The interaction of human IgG with the PC-BSA antigen was investigated using the single molecule FCS detection system. FCS data analysis was performed by fitting the autocorrelation functions using one and multiple components. The goodness-of-fit for each data set was judged by the value of the χ^2 parameter and by inspection of the residuals, which were uniformly distributed around zero. In all cases the simplest model was chosen. Attempted fits of the experimental data to a model with less independent components resulted in increased χ^2 values. Control experiments of: (i) the buffer solution, (ii) unlabeled IgG molecules in the presence of the antigen, (iii) heat-denatured labeled IgG in the presence of the PC-BSA antigen, and (iv) labeled IgG in the presence of BSA (without the PC hapten) were also performed and did not reveal a measurable interaction.

Data analysis showed that one τ_D was sufficient to describe the autocorrelation function of free IgG. Upon addition of excess antigen, three τ_D values were required for the analysis of the FCS data (Figure 6). Upon interaction of IgG with 10-fold excess PC-BSA antigen (i.e., 50μM), three different species were identified based on their diffusion time: the free, unbonded IgG molecules and those that bond to one or two antigen molecules (Figure 6). Data analysis showed that free IgG molecules have a diffusion time of ca. 1,060 μsec through the detection volume regardless of the presence of the antigen. A comparison of the

25 M.A. Leon, N.M. Young, Specificity for phosphorylcholine of six murine myeloma proteins reactive with pneumococcus C polysaccharide and B-lipoprotein, Biochemistry 10 (1971) 1424-1429.

26 L.G. Bennett, C.T. Bishop, Structure of the type XXVII *Streptococcus pneumoniae* (pneumococcal) capsular polysaccharide, Can. J. Chem. 55 (1977) 8-16.

27 C.P.J. Glaudemans, B.N. Manjula. L.G. Bennett, C.T. Bishop, Binding of phosphorylcholine-containing antigens from *Streptococcus pneumoniae* to myeloma immunoglobulins M-603 and H-8, Immunochemistry 14 (1977) 675-679.

28 D.E. Briles, J.L. Claflin, K. Schroer, C. Forman, Mouse IgG3 antibodies are highly protective against infection with *Streptococcus pneumoniae*, Nature 294 (1981) 88-90.

diffusion times of IgG before and after release through the peptide hydrogel showed that the same type of IgG-$(PC\text{-}BSA)_x$ complexes were formed upon interaction with the antigen. These complexes had diffusion times of approximately 1,135 μsec and 1,230 μsec corresponding to binding of IgG to one or two antigen molecules, respectively. Figure 6 also shows that the percentage of IgG molecules in each category (i.e., free and interacting with one or two antigen molecules), before and after release through the hydrogel was similar. These results suggest that the binding affinity of IgG for the PC-BSA antigen did not change upon release from the peptide hydrogel and therefore, the biological activity of IgG is not affected by encapsulation and release through the hydrogel.

The relatively high percentage anti-PC IgG molecules (Figure 6) in the polyclonal human IgG sample prompted further inquiry. Literature reports showed that a sizable proportion of anti-PC-BSA antibodies in the human serum, i.e., 100-800μg/ml, was also observed previously by Scott et al. using a radioimmunoassay method and blood samples from several individuals [29]. Based on these values and the concentration of IgG in the

29 M.G. Scott, D.E. Briles, P.G. Shackelford, D.S. Smith, M.H. Nahm, Human-antibodies to phosphocholine. IgG anti-PC antibodies express restricted numbers of V-regions and C-regions, J. Immunol. 138 (1987) 3325-3331.

human serum, which is ca. 8-16mg/ml, it is suggested that the relatively high percentage of anti-PC antibodies observed using the single molecule analysis is reasonable. In our experiments, the PC-BSA contains approximately 17 PC groups per BSA protein unit. This suggests that the binding observed by FluoroPoint is not due to un-specific binding but a result of high avidity of the anti-PC immunoglobulins to the PC-BSA antigen. Further work will be required to determine whether this observation can be reproduced using human IgG samples from other sources.

To test the functionality of human IgG before and after being released through the peptide scaffold hydrogel the QCM technique was also used. Criteria for IgG functionality were the kinetics of binding and the affinity constants between the monoclonal IgG and the PC-BSA antigen. Upon interaction with PC-BSA immobilized on the gold surface of the QCM, it was shown that after 48 hours, 1 month, and 2 months in the peptide hydrogel, the released human IgG did not undergo functional changes compared to the native IgG. Upon fitting of the data in Figure 7, kinetic analysis showed that the association $k_a = 9.47 \pm 0.05 \times 10^4\ M^{-1}s^{-1}$ and dissociation $k_d = 1.65 \pm 0.02 \times 10^{-3}\ s^{-1}$ rate constants for the native IgG were similar to those observed for the hydrogel released IgG $k_a = 11.85 \pm 0.10 \times 10^4\ M^{-1}s^{-1}$, $k_d = 2.39 \pm 0.02 \times 10^{-3}\ s^{-1}$. Furthermore, the binding constants, $K_D = 17.4 \pm 0.2$nM and $K_D = 20.2 \pm 0.2$nM for the native and hydrogel released IgG, respectively were similar. These results confirm the single molecule analyses and demonstrate that the functionality of the post-release antibody was not affected by interaction with the peptide hydrogel during the course of the release experiment.

CONCLUSIONS

One of the main goals of sustained drug delivery is to efficiently direct therapies to specific tissues. In cases of drug with side effects localized delivery will result in less toxicity side effects on patients. The injectable self-assembling peptide scaffold system, which gels under physiological conditions, has the potential to be a robust system for sustained release applications including immunotherapies to release active antibodies locally in specific tissues over prolonged periods of time. In this study, it is shown that human antibodies were slowly released through the ac-$(RADA)_4$-$CONH_2$ or ac-$(KLDL)_3$-$CONH_2$ peptide hydrogels for a

period of over 3 months and furthermore, the release kinetics depended on the amino acid sequence of the self-assembling peptides which form the hydrogel and the density of the peptide nanofibers in the hydrogel. An efficient controlled release system should present biologically active proteins. The secondary and tertiary structure of the hydrogel released antibodies as well as their biological activities were examined, and it was shown that they were not affected by encapsulation and release through the hydrogel. These results present an opportunity to create new tailor-made and programmable peptide hydrogels for sustained release of antibodies and other proteins. Peptide hydrogel systems can be easily designed and synthesized to control the release of IgG and other therapeutic compounds. The programmability of the peptide sequence is unique and allows a means of controlling the nanofiber properties at the molecular level, which, in turn, may alter the biomolecular diffusion and release kinetics.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims.

Peptide Sequence Listing

ac-$(RADA)_2$-$CONH_2$ (SEQ ID NO: 1)

ac-$(RADA)_3$-$CONH_2$ (SEQ ID NO: 2)

ac-$(RADA)_4$-$CONH_2$ (SEQ ID NO: 3)

ac-$(RADA)_5$-$CONH_2$ (SEQ ID NO: 4)

ac-$(KLDL)_2$-$CONH_2$ (SEQ ID NO: 5)

ac-$(KLDL)_3$-$CONH_2$ (SEQ ID NO: 6)

ac-$(KLDL)_3$-$CONH_2$ (SEQ ID NO: 7)

ac-$(KLDL)_4$-$CONH_2$ (SEQ ID NO: 8)

ac-$(KLDL)_5$-$CONH_2$ (SEQ ID NO: 9)

KLEALWLGFFGFFILGISYIR (SEQ ID NO: 10)

KLEALYIIMVLGFFGFFILGIMLSYIR (SEQ ID NO: 11)

CLAIMS

1. A sustained release formulation comprising;
   (i) a gel core comprising a gel forming first peptide and a therapeutic agent; and,
   (ii) a first layer encapsulating said gel core wherein said first layer comprises a gel forming second peptide.

2. The formulation of claim 1, wherein said formulation provides sustained release of said antibody for at least about four weeks, five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more.

3. The formulation according to claim 1, further comprising one or more layers of gel encapsulating said first layer.

4. The formulation according to any of the above claims wherein said first peptide is selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$, and Ac-$(RADA)_6$-$CONH_2$.

5. The formulation according to any of the above claims wherein said second peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$and Ac-$(KLDL)_5$-$CONH_2$.

6. The formulation according to any of the claims 1-3, wherein said second peptide is selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$, and Ac-$(RADA)_6$-$CONH_2$.

7. The formulation according to any of claims 1-2 or 6, wherein said first peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$and Ac-$(KLDL)_5$-$CONH_2$.

8. The formulation according to any of the above claims, wherein said therapeutic agent is present in the plasma of said subject upon administration for at least four weeks,

five weeks, six weeks, seven weeks, eight weeks, nine weeks, ten weeks, eleven weeks, twelve weeks or more.

9. The formulation according to any of the above claims wherein the therapeutic agent is IgD, IgA, IgM, IgE, or IgG immunoglobulin.

10. The formulation according to any of the above claims wherein said therapeutic agent is an antibody selected from the group consisting of: IgG1, IgG2, IgG3 and IgG4, IgM1 and IgM2, and IgA1 and IgA2 antibodies.

11. The formulation according to any of the above claims wherein said therapeutic agent is a whole antibody or single-chain Fv antibody fragment or Fab antibody fragment.

12. The formulation according to any of the above claims wherein said therapeutic agent is selected from Rituximab, Infliximab, Trastuzumab, Abciximab, Palivizumab, Murumonab-CD3, Gemtuzumab, Trastuzumab, Basiliximab, Daclizumab, Etanercept, and Ibritumomab tiuxetan.

13. The formulation according to any of the above claims wherein said therapeutic agent is selected from the group consisting of anti-TNF antibodies, anti-CD3 antibodies, anti-CD20 antibodies, anti-CD25 antibodies, anti-CD33 antibodies, anti-CD40 antibodies anti-HER2 antibodies, anti-HBV antibodies, anti-HAV antibodies, anti-HCV antibodies, anti-GPIIb/IIIa receptor antibodies, anti-RSV antibodies, anti-HIV antibodies, anti-HSV antibodies and anti-EBV antibodies.

14. A method of controlled delivery of a therapeutic agent comprising the step of administering a formulation according to any of the above claims to a subject in need thereof.

15. The method according to claim 14, wherein said subject is human.

16. The method according to claim 14, wherein said therapeutic agent is IgG.

17. A process for preparing a controlled release formulation comprising the steps of;
    a. preparing a first solution comprising a first self-assembling gel forming peptide, and an antibody or antibody fragment;
    b. forming a first self-assembled gel core;
    c. coating said first self-assembled gel core with a second self-assembling gel forming peptide solution; and,
    d. forming a second gel encapsulating said first self-assembled gel core.

18. The process according to claim 17, wherein said controlled release formulation further comprises the step of forming one more layers of coating comprising gel forming peptides.

19. The process according to claim 17 or 18, wherein said first self-assembling gel forming peptide is selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$, and Ac-$(RADA)_6$-$CONH_2$.

20. The process according to claim 17 or 18, wherein said second gel forming peptide is selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$, and Ac-$(RADA)_6$-$CONH_2$.

21. The process according to any of claims 17-19, wherein said second gel forming peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$ and Ac-$(KLDL)_5$-$CONH_2$.

22. The process according to any of claims 17 or 18, wherein said first gel forming peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$ and Ac-$(KLDL)_5$-$CONH_2$.

23. The process according to claim 16, wherein said one or more layers comprises a gel forming peptide selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$, Ac-$(KLDL)_5$-$CONH_2$, Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$ and Ac-$(KLDL)_5$-$CONH_2$.

24. The process according to any of claims 17-23, wherein the antibody is an IgD, IgA, IgM, IgE, or IgG immunoglobulin.

25. The process according claim 24, wherein said antibody is selected from the group consisting of: IgG1, IgG2, IgG3 and IgG4, IgM1 and IgM2, and IgA1 and IgA2 antibodies.

26. A sustained release formulation prepared by a process according to any of claims 17-25.

27. A process for preparing a controlled release formulation comprising the steps of;
    a. preparing a first solution comprising a first self-assembling gel forming peptide, and a therapeutic agent;
    b. forming a first self-assembled gel core;
    c. coating said gel core with a second self-assembling gel forming peptide; and,
    d. forming a second gel encapsulating said gel core.

28. The process according to claim 27, wherein said controlled release formulation further comprises the step of forming one more layers of coating comprising gel forming peptides.

29. The process according to claim 27 or 28, wherein said first self-assembling gel forming peptide is selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$, and Ac-$(RADA)_6$-$CONH_2$.

30. The process according to claim 27 or 28, wherein said second gel forming peptide is selected from Ac-$(RADA)_3$-$CONH_2$, Ac-$(RADA)_4$-$CONH_2$, Ac-$(RADA)_5$-$CONH_2$, and Ac-$(RADA)_6$-$CONH_2$.

31. The process according to any of claims 27-28, wherein said first gel forming peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$ and Ac-$(KLDL)_5$-$CONH_2$.

32. The process according to any of claim 17, wherein said first gel forming peptide is selected from Ac-$(KLDL)_3$-$CONH_2$, Ac-$(KLDL)_4$-$CONH_2$ and Ac-$(KLDL)_5$-$CONH_2$.

33. The process according to any of claims 27-32, wherein said therapeutic agent is a protein.

34. The process according to any of claims 27-32, wherein said therapeutic agent is selected from erythropoietin (EPO), interferon-alpha, interferon-beta, interferon-gamma, growth hormone, growth hormone releasing factor, nerve growth factor (NGF), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), macrophage-colony stimulating factor (M-CSF), blood clotting factor, insulin, oxytocin, vasopressin, adrenocorticotropic hormone, epidermal growth factor, platelet-derived growth factor (PDGF), prolactin, luliberin, luteinizing hormone releasing hormone (LHRH), LHRH agonists, LHRH antagonists, somatostatin, glucagon, interleukin-2 (IL-2), interleukin-11 (IL-11), gastrin, tetragastrin, pentagastrin, urogastrone, secretin, calcitonin, enkephalins, endorphins, angiotensins, thyrotropin releasing hormone (TRH), tumor necrosis factor (TNF), tumor necrosis factor related apoptosis inducing ligand (TRAIL), heparinase, bone morphogenic protein (BMP), human atrial natriuretic peptide (hANP), glucagon-like peptide (GLP-1), renin, bradykinin, bacitracins, polymyxins, colistins, tyrocidine, gramicidins, cyclosporins, small interference RNA (siRNA), plasmid DNA, and antisense oligodeoxynucleotide (AS-ODN).

35. A sustained release formulation prepared by a process according to any of claims 27-34.

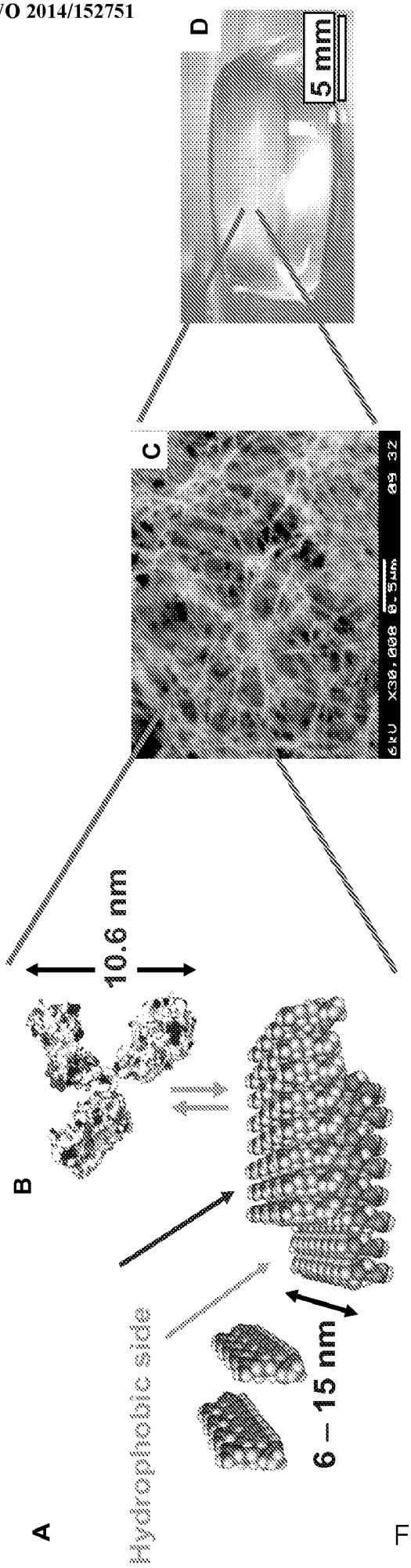


FIGURE 1

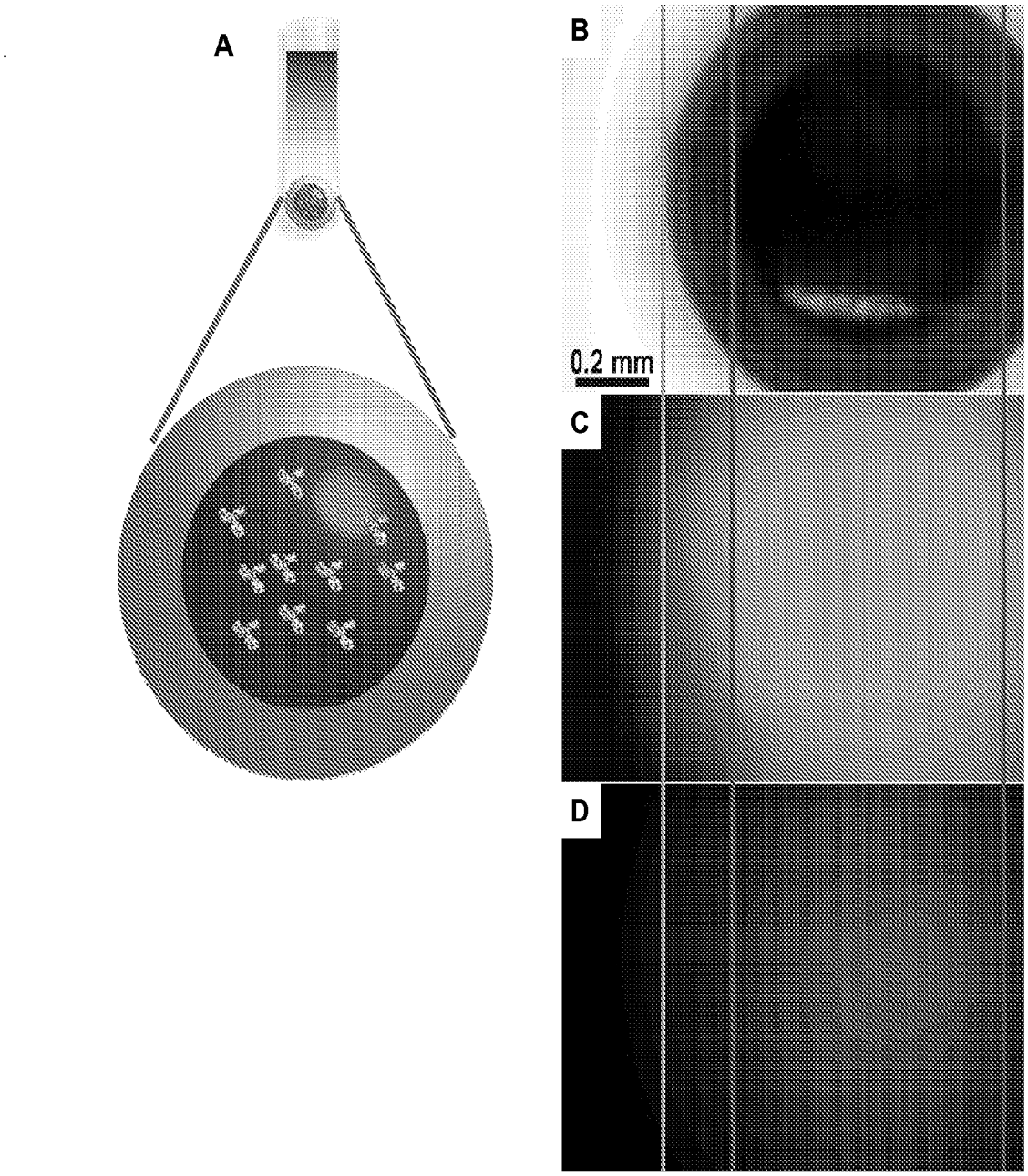


Figure 2

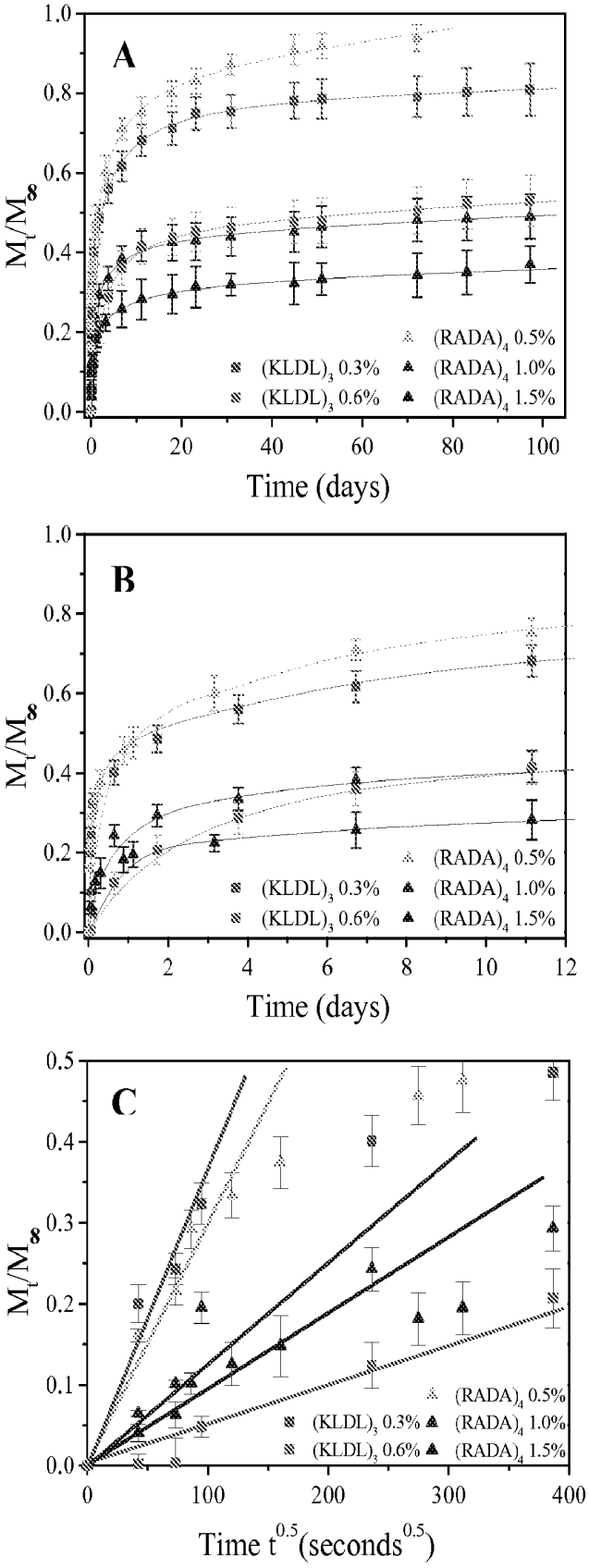

A
M_t/M_8
Time (days)
$(KLDL)_3$ 0.3%
$(KLDL)_3$ 0.6%
$(RADA)_4$ 0.5%
$(RADA)_4$ 1.0%
$(RADA)_4$ 1.5%
B
M_t/M_8
Time (days)
$(KLDL)_3$ 0.3%
$(KLDL)_3$ 0.6%
$(RADA)_4$ 0.5%
$(RADA)_4$ 1.0%
$(RADA)_4$ 1.5%
C
M_t/M_8
Time $t^{0.5}$ (seconds$^{0.5}$)
$(KLDL)_3$ 0.3%
$(KLDL)_3$ 0.6%
$(RADA)_4$ 0.5%
$(RADA)_4$ 1.0%
$(RADA)_4$ 1.5%

Figure 3

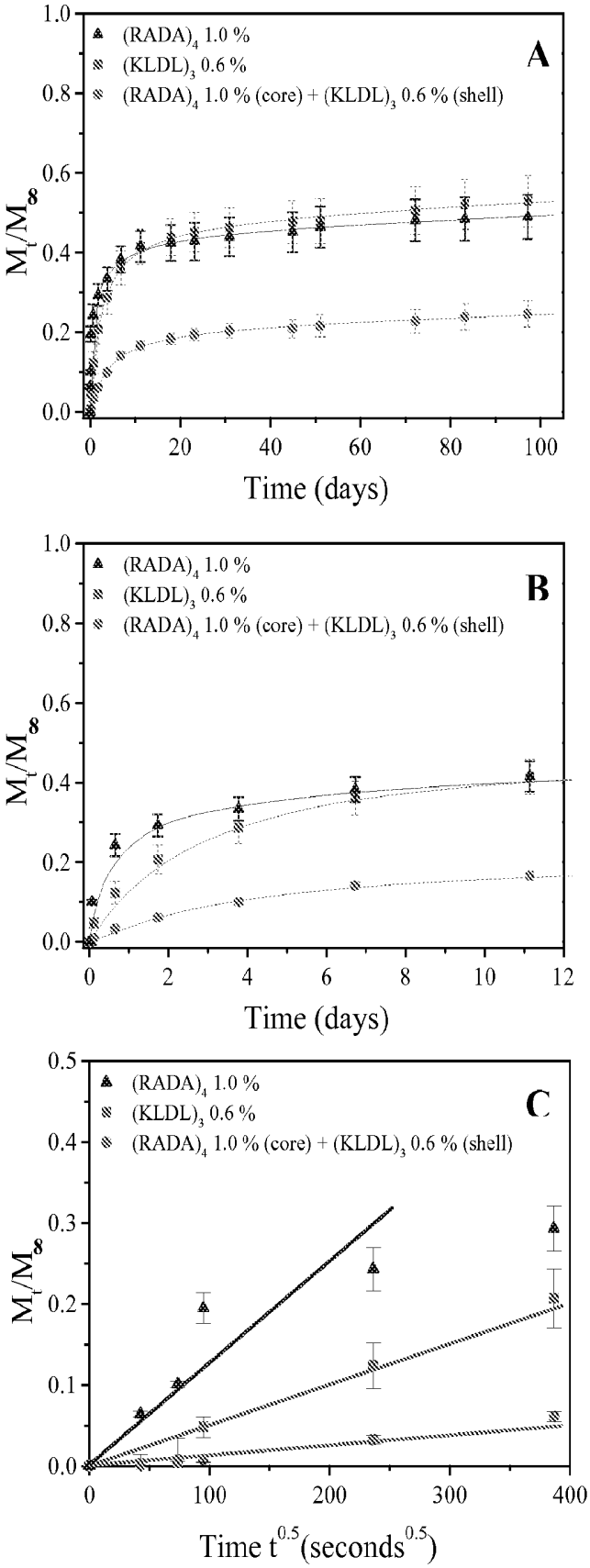

A
(RADA)4 1.0 %
(KLDL)3 0.6 %
(RADA)4 1.0 % (core) + (KLDL)3 0.6 % (shell)
Mt/M∞
Time (days)
B
(RADA)4 1.0 %
(KLDL)3 0.6 %
(RADA)4 1.0 % (core) + (KLDL)3 0.6 % (shell)
Mt/M∞
Time (days)
C
(RADA)4 1.0 %
(KLDL)3 0.6 %
(RADA)4 1.0 % (core) + (KLDL)3 0.6 % (shell)
Mt/M∞
Time t0.5 (seconds0.5)

Figure 4

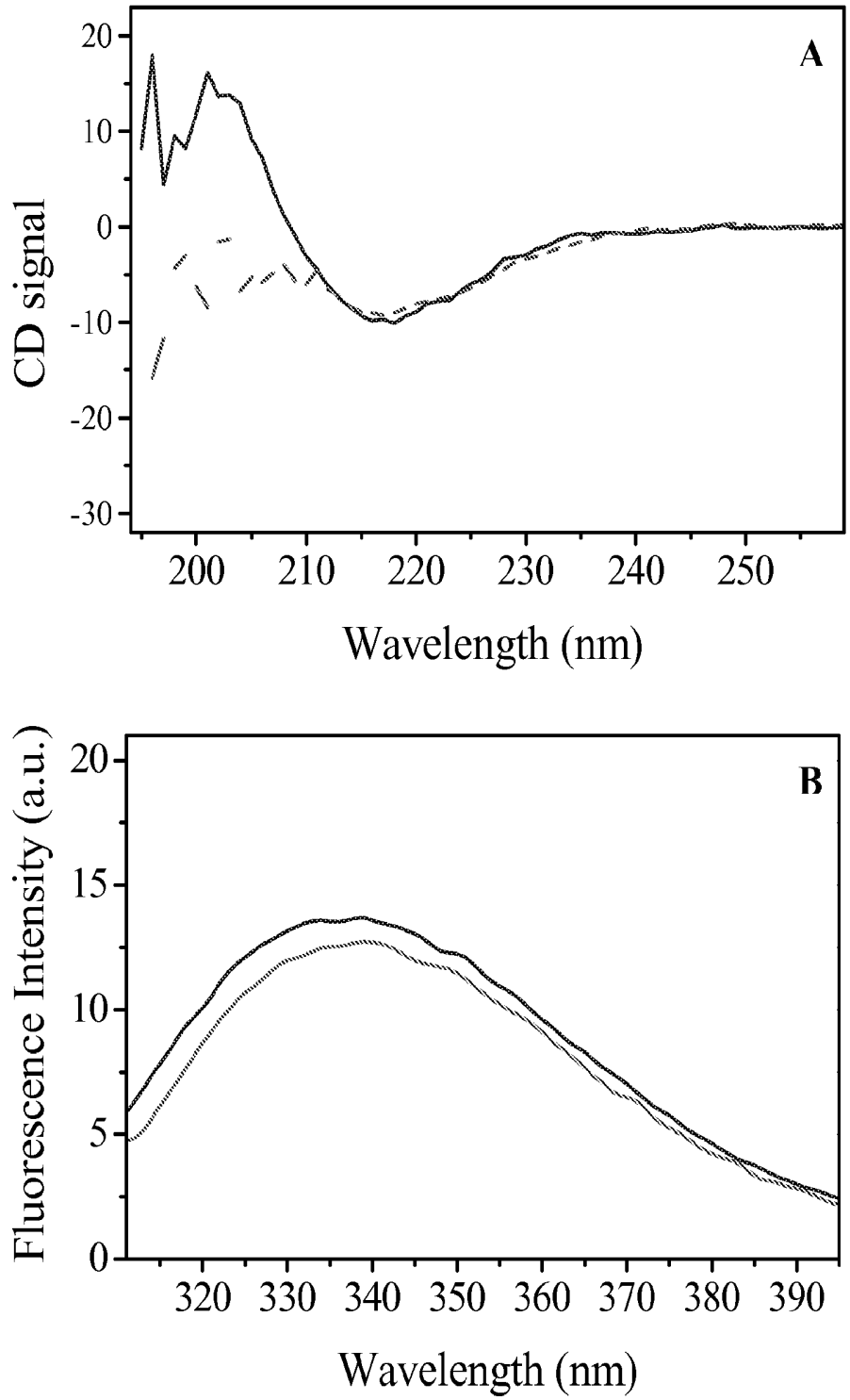

A
CD signal
20
10
0
-10
-20
-30
200
210
220
230
240
250
Wavelength (nm)
B
Fluorescence Intensity (a.u.)
20
15
10
5
0
320
330
340
350
360
370
380
390
Wavelength (nm)

Figure 5

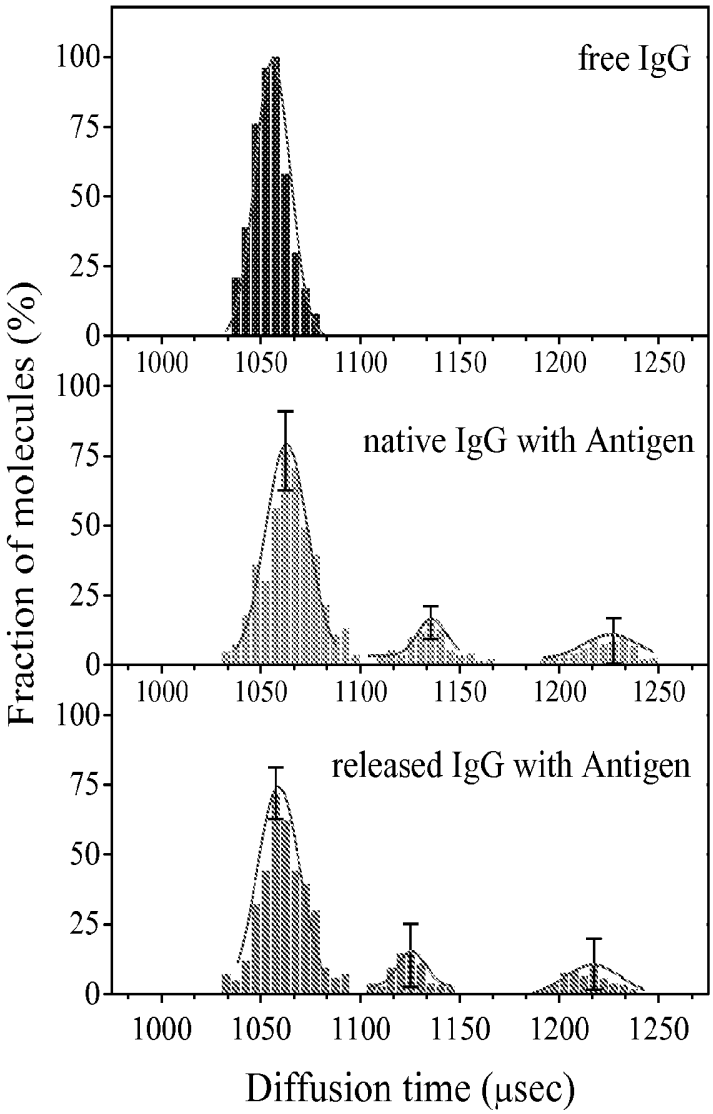

free IgG
native IgG with Antigen
released IgG with Antigen
Fraction of molecules (%)
Diffusion time (μsec)
0
25
50
75
100
1000
1050
1100
1150
1200
1250

Figure 6

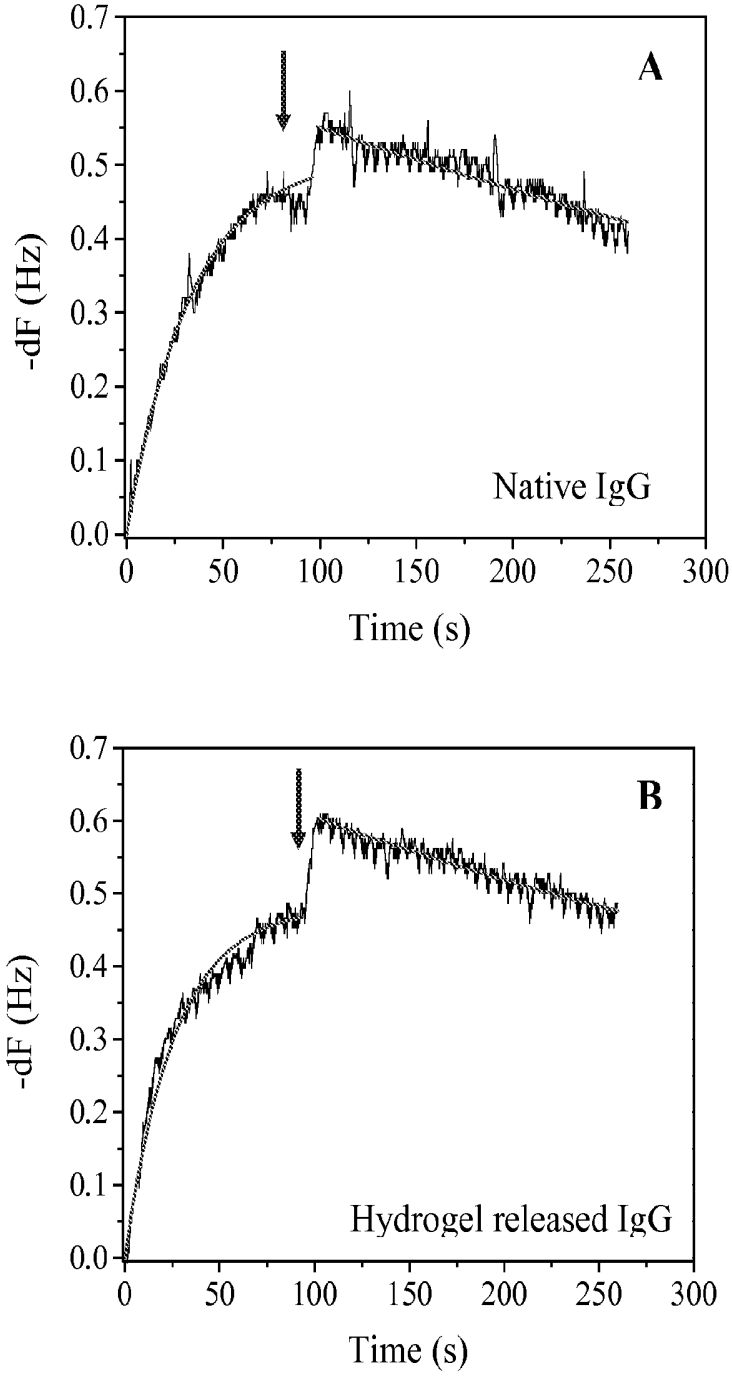

A
Native IgG
-dF (Hz)
Time (s)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0
50
100
150
200
250
300
B
Hydrogel released IgG
-dF (Hz)
Time (s)

Figure 7

Peptide hydrogel	hydrogel density (w/v)	Diffusion constants (10^{-10} m^2/s)	
		Stokes-Einstein estimate	Apparent in gel (Equation 1)
ac-$(RADA)_4$-$CONH_2$	0.5%	0.4	0.183 ± 0.002
	1.0%		0.028 ± 0.001
	1.5%		0.017 ± 0.001
ac-$(KLDL)_3$-$CONH_2$	0.3%	0.4	0.205 ± 0.004
	0.6%		0.0040 ± 0.0001
ac-$(RADA)_4$-$CONH_2$ (core) + ac-$(KLDL)_3$-$CONH_2$ (shell)	1.0% (core) + 0.6% (shell)	0.4	0.0010 ± 0.0001

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/27693

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A61K 9/10, 38/00; C07K 7/00, 16/00 (2014.01)
USPC - 424/484; 530/300, 387.1, 817; 977/915, 918
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): A61K 9/10, 38/00; C07K 7/00, 16/00; C12N 5/02, 15/86 (2014.01)
USPC - 424/484; 435/397, 457, 456, 400, 451, 464, 468; 514/12; 530/300, 387.1, 386, 380, 350, 817; 977/915, 918

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google; Google Scholar; PubMed; 'gel forming peptide,' 'amphiphile peptide,' encapsulate, layers, 'sustained release/delivery,' antibody, 'Ac-(RADA)3-CONH2,' 'Ac-(RADA) 4-CONH2,' 'Ac-(KLDL)3-CONH2,' 'Ac-(KLDL)4-CONH2'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOUTSOPOULOS, S, et al. Two-Layered Injectable Self-Assembling Peptide Scaffold Hydrogels For Long-Term Sustained Release Of Human Antibodies. J. Control Release. 28 June 2012. Vol. 160, No. 3: pages 451-458; abstract; page 452, column 1, paragraph 3; page 452, column 2, paragraphs 5-7; figures 2, 3; page 454, column 2, paragraph 6; figure 4A; page 455, column 2, paragraph 5.	1-3, 4/1-4/3, 6/1-6/3, 17, 18, 19/17, 19/18, 20/17, 20/18, 22/17, 22/18, 27, 28, 29/27, 29/28, 30-32
A	US 2009/0162437 A1 (HORII, A et al.) June 25, 2009; abstract; figure 36; paragraphs [0186], [0205], [0206], [0212], [0213], [0316], [0318], [0319], [0329], [0428], [0429]; SEQ ID NOs: 21, 53	1-4, 6/1-6/3, 17-20, 22/17, 22/18, 27-32

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 August 2014 (04.08.2014)	22 AUG 2014

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/27693

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5, 7-16, 21, 23-26, 33-35
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

摘要

本發明涉及一種用於持續傳遞治療劑的藥物製劑，所述治療劑優選是蛋白質、多肽、抗體或者抗體片段，所述藥物製劑包括一種或者一種以上凝膠形成肽，其中，所述製劑在至少兩週、至少三週、至少四周、至少五週、至少六週、至少七週、至少八週、至少九週、至少十週、至少十一周和至少十二週或更長的時間內顯示持續釋放。在一個實施方案中，本發明涉及一種製劑，所述製劑包括自組裝肽，該肽在電解質溶液，例如生物液體和鹽存在的情況下經過溶膠-凝膠轉變。所述製劑提供抗體和抗體片段的持續釋放，尤其是IgG。增加水凝膠納米纖維密度可以減少抗體擴散，提供了一種控制釋放動力學的手段。