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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, 5 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 10 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) 15 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 20 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-CONH₂ (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-CONH₂ 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. Furthermore they are non-toxic, non-immunogenic, non-thrombogenic, biodegradable, and applicable to localized therapies through injection to a particular tissue [14,15].

5 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
10 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 biodegradable peptides that can provide delivery of
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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-CONH₂ core and ac-(KLDL)₃-CONH₂ 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-CONH₂ and ac-(KLDL)₃-CONH₂ 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-CONH₂ or ac-(KLDL)₃-CONH₂ 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.

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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.

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Figure 1. Graphical representation of (A) the ac-(RADA)₄-CONH₂ 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).

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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-CONH₂ peptide) is loaded with the Alexa-488 fluorophore (C) and the core (self-assembled ac-(RADA)₄-CONH₂ peptide) is loaded with CY3 (D).

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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 assembling peptides (i) ac-(RADA)₄-CONH₂ 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-CONH₂ 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).

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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-CONH₂ with concentration of 1.0 % w/v (blue, ▲), ac-(KLDL)₃-CONH₂ with concentrations 0.6 % w/v (magenta, ■), and multi-layered, two-component hydrogel which is composed of 1.0 % w/v ac-(RADA)₄-CONH₂ (core) and 0.6 % w/v ac-(KLDL)₃-CONH₂ (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

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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-CONH₂ 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 $r_h = 5.3\text{nm}$, 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 5 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 10 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-CONH₂ core and ac-(KLDL)₃-CONH₂ shell. The antibody can be diffused through ‘onion-like’ architectures that can be formed when complementary peptides are used. The sustained 15 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 20 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 25 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 30 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-CONH₂ and ac-(KLDL)₃-CONH₂ 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-CONH₂ core and ac-(KLDL)₃-CONH₂ 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-CONH₂ or ac-(KLDL)₃-CONH₂ 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 5 preferred embodiment, the core comprises a first peptide selected from Ac-(RADA)₃-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂; and a said peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂, Ac-(KLDL)₅-CONH₂; 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-CONH₂ peptide solution (about 0.2 to 10 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 1 μ M to about 20 μ M; preferably between about 2 μ M to about 10 μ M; 4 μ M to about 8 μ M) a gel containing IgG can be formed. The gel can be 15 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-CONH₂ can be applied on the outer surface of the core. The ac-(KLDL)₃-CONH₂ peptide 20 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-CONH₂ peptide 25 solution to come in contact and encapsulate the preformed ac-(RADA)₄-CONH₂ 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)₄CONH₂/IgG core coated with ac-(KLDL)₃-CONH₂ can be further coated with a layer of ac-(RADA)₄CONH₂/IgG followed by another coating of ac-(KLDL)₃-CONH₂ based on the 30 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 5 described in Boden et al. (U.S. Patent Publication 20020132974); or PF4₂₄₋₄₆ 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.

10 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 released over a period of at least about one month, or two months or three months.

15 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.

20 "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.

25 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 30 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., 5 *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 10 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 15 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 20 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 25 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 30 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 5 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 10 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.

15 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 20 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 25 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, 30 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, 5 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, 10 the invention relates to formulations comprising a therapeutic agent selected from insulin, leuprolide, gonadorelin, somatostatin, octreotide, enfuvirtide, 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 15 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 20 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, 25 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 30 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, meseclazole, 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, seclazole, 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 sulfate; 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, nericin (penta-erythritol tetranicotinate), nicofuranose and nicotinyl alcohol; a bile acid sequestrant, including colestevolam, colestyramine, colestipol

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

5 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

10 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 15 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 20 (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), 25 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.

30 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-CONH₂ and ac-(KLDL)₃-CONH₂ 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-CONH₂ and ac-(KLDL)₃-CONH₂ 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-CONH₂ 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-CONH₂ 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-CONH₂ peptide solution to come in contact and

5 encapsulate the preformed ac-(RADA)₄-CONH₂ 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

10 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

15 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-CONH₂ and the ac-(RADA)₄-CONH₂ peptide solutions with the Alexa-488 (green) and CY3 (red) fluorescent dyes solutions, respectively. The multi-layered hydrogel consists of two concentric spheres

20 with composition 1.0% w/v ac-(RADA)₄-CONH₂ (core) and 0.6% w/v ac-(KLDL)₃-CONH₂ (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

25 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]:

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

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

30 thickness of the hydrogel matrix (H) inside the well was calculated from the volume of the

peptide-IgG solution (i.e., 40 μ l) 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 $0 < M_t/M_\infty < 0.6$.

5 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
10 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
15 2.5nm, respectively. Sample conditions were identical to those described for the CD measurements.

20 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
25 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 5 Tween concentration.

Kinetic data at room temperature were obtained by injecting 35 μ L of 20 μ g/mL human IgG for 84 seconds at 20 μ L/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 10 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, 15 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 20 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 25 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 30 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-
5 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-CONH₂ 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-CONH₂ peptide hydrogels with concentrations of
10 0.3% w/v (99.7% water) and 0.6% w/v (99.4% water). Higher density ac-(KLDL)₃-CONH₂ 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
15 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-CONH₂ peptide and contains the CY3 dye (red) whereas the encapsulating second sphere is composed by ac-(KLDL)₃-CONH₂ and contains the
20 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-CONH₂ and the ac-(RADA)₄-CONH₂ hydrogels, respectively and homogeneous distribution throughout the entire two-component peptide hydrogel system is expected, the initial distribution of the dyes clearly defines the
25 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-CONH₂ peptide is loaded with the antibody whereas the second layer (shell) consists of the ac-(KLDL)₃-CONH₂ hydrogel does not contain antibody and it encapsulates the core. The formation of the multilayered hydrogel resulted in a system in which the initial
30 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 $0.4 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ 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

²⁰ J.K. Armstrong, R.B. Wenby, H.J. Meiselman, T.C. Fisher, The hydrodynamic radii of macromolecules and their effect on red blood cell aggregation, *Biophys. J.* 87 (2004) 4259–4270.

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
5 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
10 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
15 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.

20 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
25 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

21 J. Crank, G.S. Park, *Diffusion in polymers*, Academic Press, NY, 1968.

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., 50 μ M), 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 μ sec 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-BSA)_x complexes were formed upon interaction with the antigen. These complexes had diffusion times of approximately 1,135 μ sec and 1,230 μ sec corresponding to binding of IgG to one or two antigen molecules, respectively. Figure 6 also 5 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.

10 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., 100-800 μ g/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 5 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 10 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 $k_a = 9.47 \pm 0.05 \times$ 15 $10^4 \text{ M}^{-1} \text{s}^{-1}$ and dissociation $k_d = 1.65 \pm 0.02 \times 10^{-3} \text{ s}^{-1}$ rate constants for the native IgG were similar to those observed for the hydrogel released IgG $k_a = 11.85 \pm 0.10 \times 10^4 \text{ M}^{-1} \text{s}^{-1}$, $k_d = 2.39 \pm 0.02 \times 10^{-3} \text{ s}^{-1}$. Furthermore, the binding constants, $K_D = 17.4 \pm 0.2 \text{nM}$ and $K_D = 20.2 \pm 0.2 \text{nM}$ for the native and hydrogel released IgG, respectively were similar. These results 20 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 25 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-CONH₂ or ac-(KLDL)₃-CONH₂ 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 5 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 10 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 15 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-CONH₂ (SEQ ID NO: 1)
20 ac-(RADA)₃-CONH₂ (SEQ ID NO: 2)
ac-(RADA)₄-CONH₂ (SEQ ID NO: 3)
ac-(RADA)₅-CONH₂ (SEQ ID NO: 4)
ac-(KLDL)₂-CONH₂ (SEQ ID NO: 5)
ac-(KLDL)₃-CONH₂ (SEQ ID NO: 6)
25 ac-(KLDL)₃-CONH₂ (SEQ ID NO: 7)
ac-(KLDL)₄-CONH₂ (SEQ ID NO: 8)
ac-(KLDL)₅-CONH₂ (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-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂, and Ac-(RADA)₆-CONH₂.
5. The formulation according to any of the above claims wherein said second peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.
6. The formulation according to any of the claims 1-3, wherein said second peptide is selected from Ac-(RADA)₃-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂, and Ac-(RADA)₆-CONH₂.
7. The formulation according to any of claims 1-2 or 6, wherein said first peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.
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
5 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.

10

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.

15

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.

20

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.

25

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.

30

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;
- 5 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

10 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-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂, and Ac-(RADA)₆-CONH₂.

20. The process according to claim 17 or 18, wherein said second gel forming peptide is selected from Ac-(RADA)₃-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂, and Ac-(RADA)₆-CONH₂.

20
21. The process according to any of claims 17-19, wherein said second gel forming peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.

25
22. The process according to any of claims 17 or 18, wherein said first gel forming peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.

30
23. The process according to claim 16, wherein said one or more layers comprises a gel forming peptide selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂, Ac-(KLDL)₅-CONH₂, Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.

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

5 25. The process according to 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.

10 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.

15 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.

20 29. The process according to claim 27 or 28, wherein said first self-assembling gel forming peptide is selected from Ac-(RADA)₃-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂, and Ac-(RADA)₆-CONH₂.

25 30. The process according to claim 27 or 28, wherein said second gel forming peptide is selected from Ac-(RADA)₃-CONH₂, Ac-(RADA)₄-CONH₂, Ac-(RADA)₅-CONH₂, and Ac-(RADA)₆-CONH₂.

31. The process according to any of claims 27-28, wherein said first gel forming peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.
- 5 32. The process according to any of claim 17, wherein said first gel forming peptide is selected from Ac-(KLDL)₃-CONH₂, Ac-(KLDL)₄-CONH₂ and Ac-(KLDL)₅-CONH₂.
33. The process according to any of claims 27-32, wherein said therapeutic agent is a protein.
- 10 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).
- 20 35. A sustained release formulation prepared by a process according to any of claims 27-34.
- 25 30

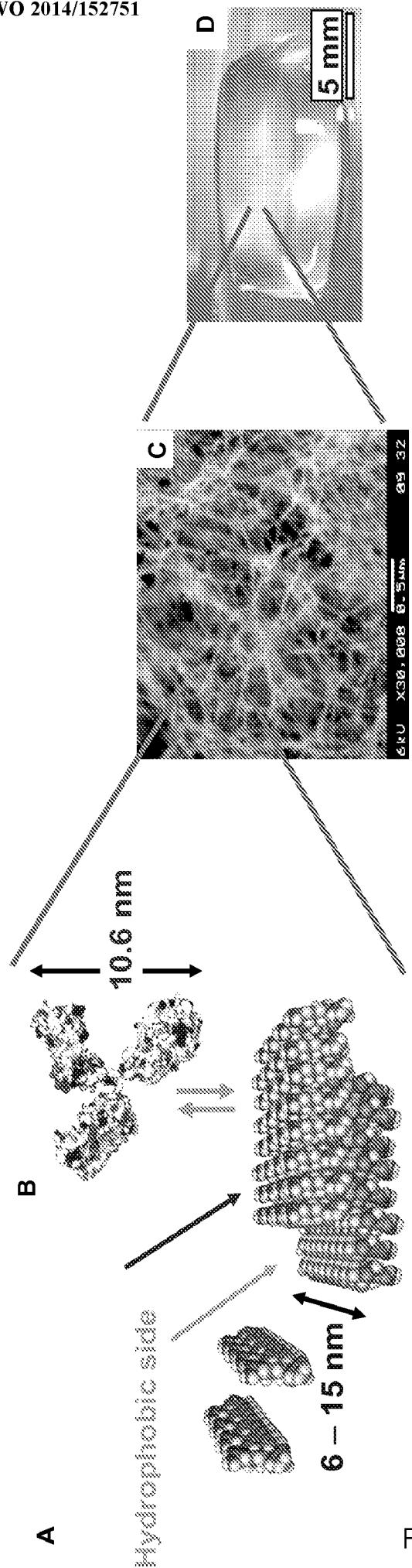


FIGURE 1

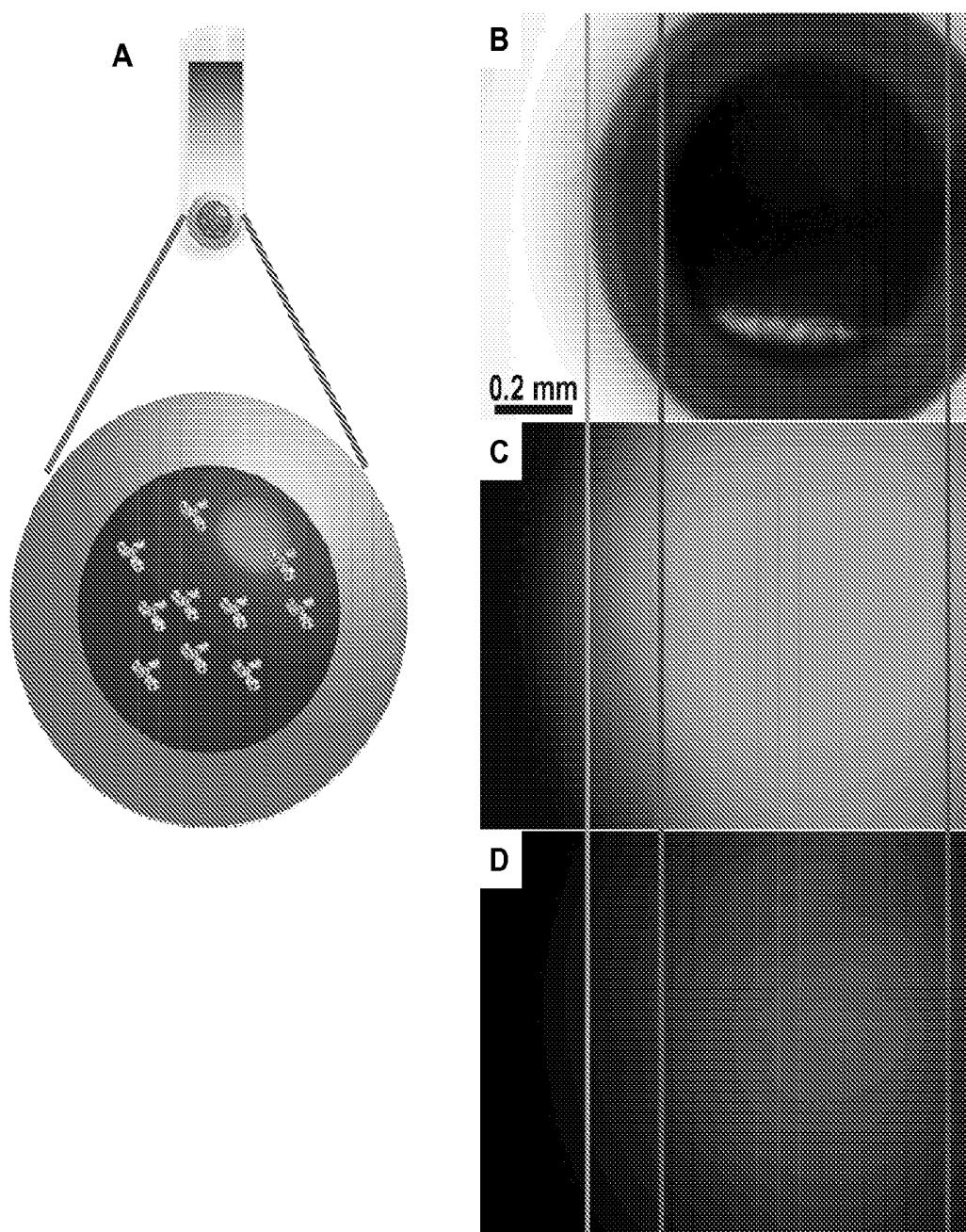
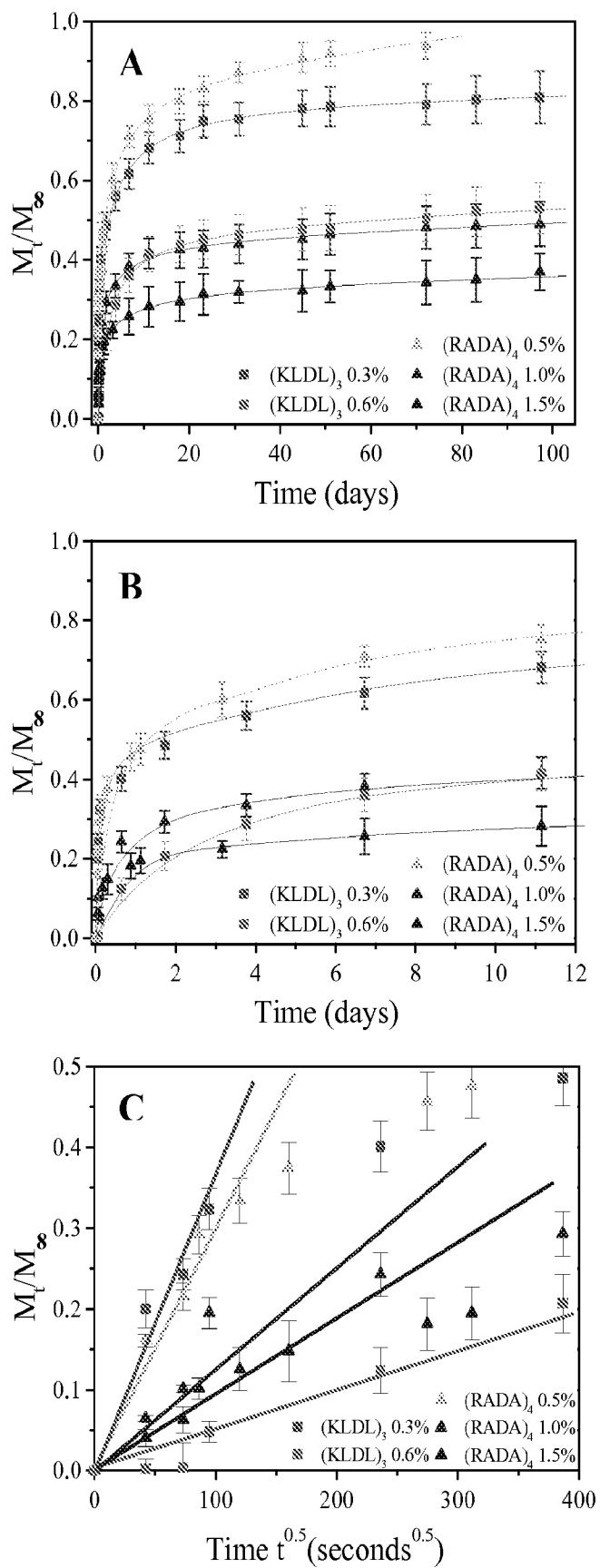
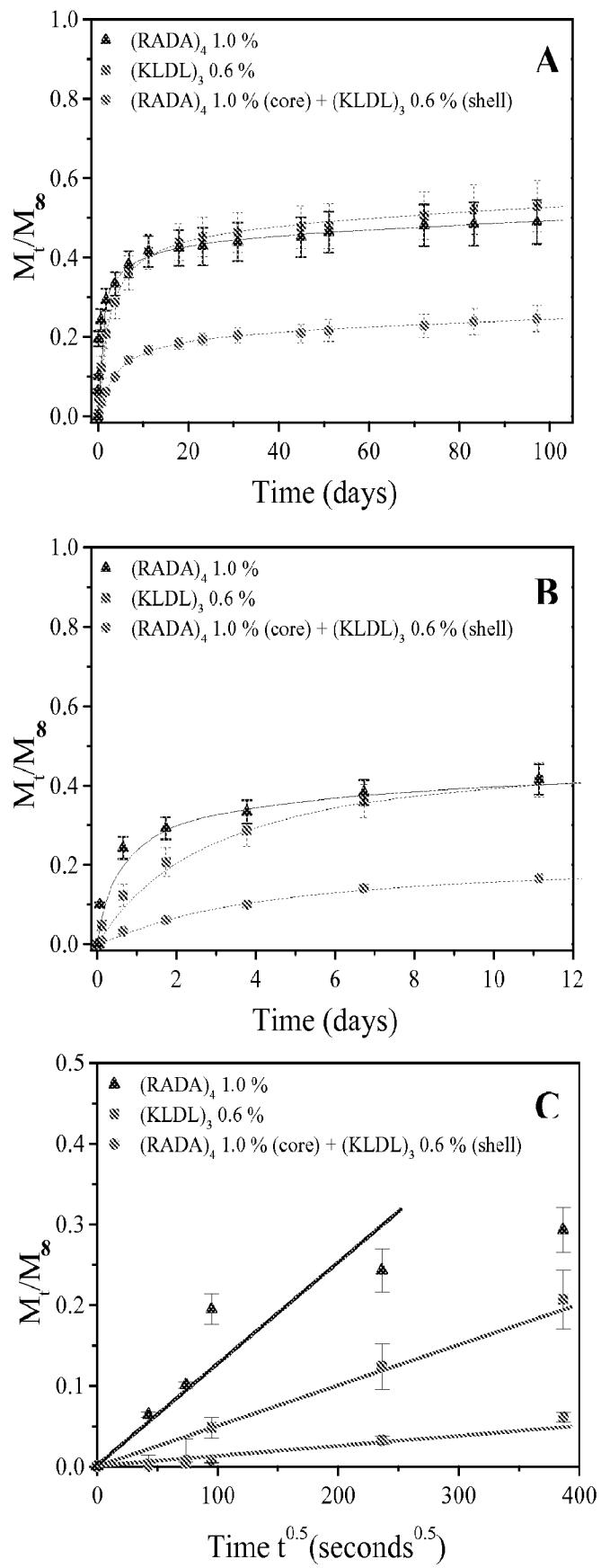


Figure 2

**Figure 3**

**Figure 4**

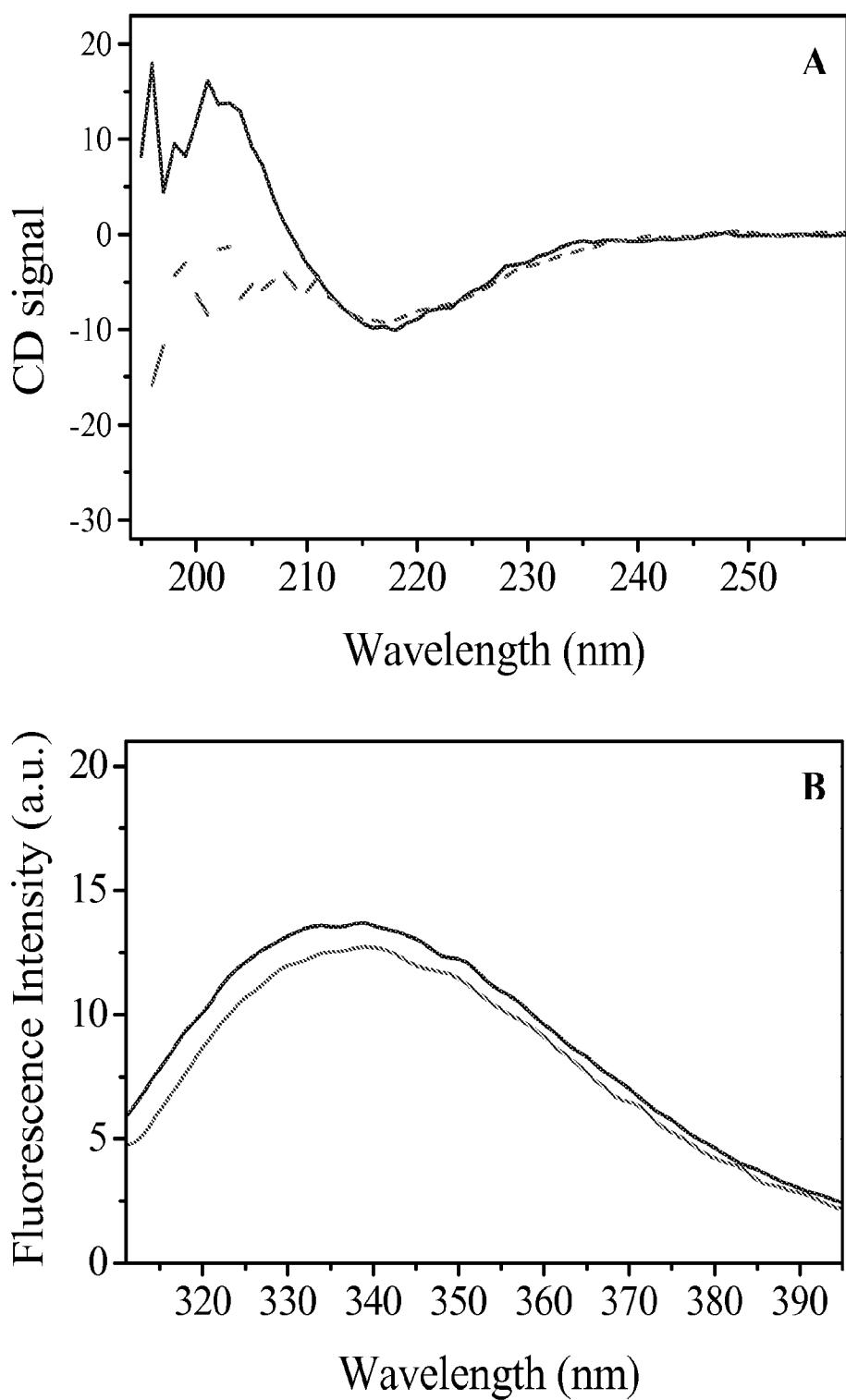


Figure 5

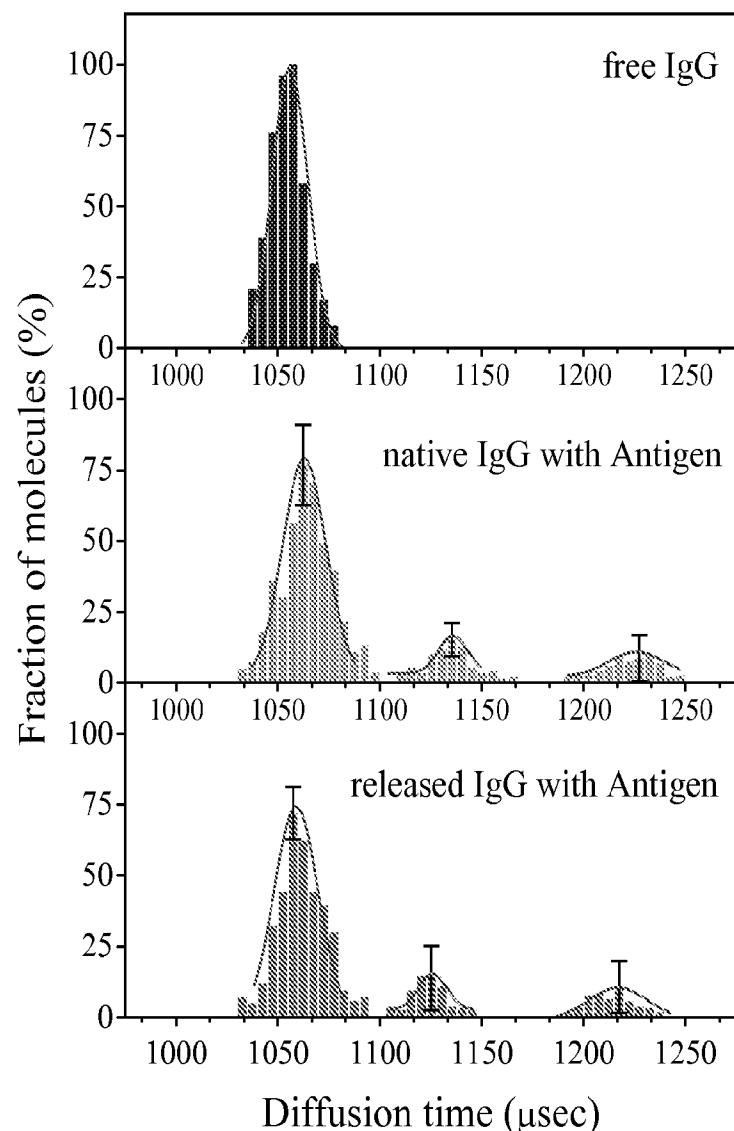


Figure 6

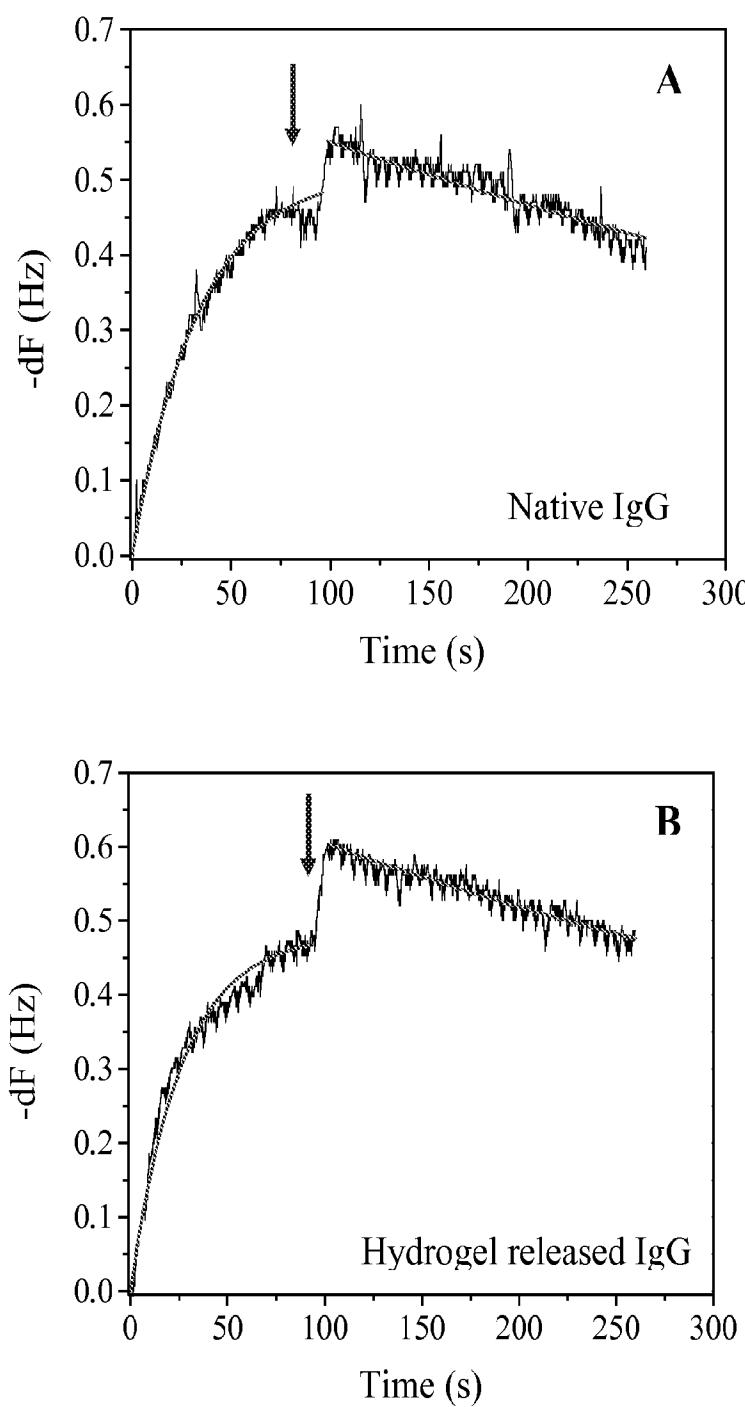


Figure 7

Peptide hydrogel	hydrogel density (w/v)	Diffusion constants (10^{-10} m ² /s)	
		Stokes-Einstein estimate	Apparent in gel (Equation 1)
	0.5%		0.183 ± 0.002
ac-(RADA) ₄ -CONH ₂	1.0%	0.4	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)	1.0% (core)		
+	+	0.4	0.0010 ± 0.0001
ac-(KLDL) ₃ -CONH ₂ (shell)	0.6% (shell)		

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. <i>J. Control Release</i> . 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 04 August 2014 (04.08.2014)	Date of mailing of the international search report 22 AUG 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

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.



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72002

权利要求书3页 说明书17页 附图7页

(54) 发明名称

用于长时间持续释放人类抗体的多层可注射
自组装肽脚手架水凝胶

(57) 摘要

本发明涉及一种用于持续传递治疗剂的药物制剂，所述治疗剂优选是蛋白质、多肽、抗体或者抗体片段，所述药物制剂包括一种或者一种以上凝胶形成肽，其中，所述制剂在至少两周、至少三周、至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周、至少十一周和至少十二周或更长的时间内显示持续释放。在一个实施方案中，本发明涉及一种制剂，所述制剂包括自组装肽。该肽在电解质溶液，例如生物液体和盐存在的条件下经过溶胶-凝胶转变。所述制剂提供抗体和抗体片段的持续释放，尤其是 IgG。增加水凝胶纳米纤维密度可以减少抗体扩散，提供了一种控制释放动力学的手段。

1. 一种持续释放制剂, 包括 :
 - (i) 一种凝胶核心, 其包括由第一肽和一种治疗剂形成的凝胶 ; 和
 - (ii) 第一层, 包覆所述凝胶核心, 其中, 所述第一层包括第二肽形成的凝胶。
2. 根据权利要求 1 所述的制剂, 其中, 所述制剂在至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周、至少十一周和至少十二周或更长的时间内持续释放所述抗体。
3. 根据权利要求 1 所述的制剂, 进一步包括包覆所述第一层的一个或者多个凝胶层。
4. 根据前述任意一项所述的制剂, 其中, 所述第一肽选自 $\text{Ac}-(\text{RADA})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_5-\text{CONH}_2$ 和 $\text{Ac}-(\text{RADA})_6-\text{CONH}_2$ 。
5. 根据前述任意一项所述的制剂, 其中, 所述第二肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。
6. 根据权利要求 1-3 中任意一项所述的制剂, 其中, 所述第二肽选自 $\text{Ac}-(\text{RADA})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_5-\text{CONH}_2$ 和 $\text{Ac}-(\text{RADA})_6-\text{CONH}_2$ 。
7. 根据权利要求 1-2, 6 中任意一项所述的制剂, 其中, 所述第一肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。
8. 根据前述任意一项权利要求所述的制剂, 其中, 所述治疗剂随着给药存在于所述主体的血浆中至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周、至少十一周和至少十二周或更长的时间。
9. 根据前述任意一项权利要求所述的制剂, 其中, 所述治疗剂是 IgD、IgA、IgM、IgE 或者 IgG 免疫球蛋白。
10. 根据前述任意一项权利要求所述的制剂, 其中, 所述治疗剂是抗体, 所述抗体选自由 IgG1、IgG2、IgG3 和 IgG4, IgM1 和 IgM2, 和 IgA1 和 IgA2 所组成的组中。
11. 根据前述任意一项权利要求所述的制剂, 其中, 所述治疗剂是全抗体或者单链 Fv 抗体片段或者 Fab 抗体片段。
12. 根据前述任意一项权利要求所述的制剂, 所述治疗剂选自美罗华、英夫利昔单抗、曲妥珠单抗、阿昔单抗、帕利珠单抗、Murumonab-CD3、吉妥单抗、曲妥珠单抗、巴利昔单抗、达(克)珠单抗、依那西普和替伊莫单抗、伊莫单抗。
13. 根据前述任意一项权利要求所述的制剂, 所述治疗剂选自由抗肿瘤坏死因子抗体、抗-CD3 抗体、抗-CD20 抗体、抗-CD25 抗体、抗-CD33 抗体、抗-CD40 抗体、抗-HER2 抗体、抗-HBV 抗体、抗 HAV 抗体、抗 HCV 抗体、抗 GPIIb/IIIa 受体抗体、抗 RSV 抗体、抗 HIV 抗体、抗 HSV 抗体和抗 EBV 抗体所组成的组中。
14. 一种控制治疗剂传递的方法, 包括向需要的主体给药前述任意一项权利要求所述的制剂的步骤。
15. 根据权利要求 14 所述的方法, 其中, 所述主体是人。
16. 根据权利要求 14 所述的方法, 其中, 所述治疗剂是 IgG。
17. 制备控制释放制剂的过程, 包括以下步骤 :
 - a. 制备第一溶液, 包括由第一自组装凝胶形成肽和抗体或者抗体片段 ;
 - b. 形成第一自组装凝胶核心 ;
 - c. 用第二自组装凝胶形成肽涂布所述第一自组装凝胶核心 ; 并且

d. 形成包覆所述第一自组装凝胶核心的第二凝胶。

18. 根据权利要求 17 所述的过程, 其中, 所述控制释放制剂进一步包括形成一个或者多个包覆层的步骤, 所述包覆层包括凝胶形成肽。

19. 根据权利要求 17 或 18 所述的过程, 其中, 所述第一自组装凝胶形成肽选自 $\text{Ac}-(\text{RADA})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_5-\text{CONH}_2$ 和 $\text{Ac}-(\text{RADA})_6-\text{CONH}_2$ 。

20. 根据权利要求 17 或 18 所述的过程, 其中, 所述第二凝胶形成肽选自 $\text{Ac}-(\text{RADA})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_5-\text{CONH}_2$ 和 $\text{Ac}-(\text{RADA})_6-\text{CONH}_2$ 。

21. 根据权利要求 17-19 中任意一项所述的过程, 其中, 所述第二凝胶形成肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。

22. 根据权利要求 17 或 18 所述的过程, 其中, 所述第一凝胶形成肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。

23. 根据权利要求 16 所述的过程, 其中, 所述一个或者更多层包括凝胶形成肽, 所述凝胶形成肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。

24. 根据权利要求 17-23 中任意一项所述的过程, 其中, 所述抗体是 IgD、IgA、IgM、IgE 或者 IgG 免疫球蛋白。

25. 根据权利要求 24 所述的过程, 其中, 所述抗体选自由 IgG1, IgG2, IgG3 和 IgG4, IgM1 和 IgM2, 和 IgA1 和 IgA2 抗体所组成的组中。

26. 根据权利要求 17-25 中任意一项所述的过程制备的持续释放制剂。

27. 一种制备控制释放制剂的过程, 包括以下步骤 :a. 制备第一溶液, 所述第一溶液包括第一自组装凝胶形成肽和治疗剂 ;b. 形成第一自组装凝胶核心 ;c. 用第二自组装凝胶形成肽涂布所述凝胶核心 ; 和 d. 形成包覆所述凝胶核心的第二凝胶。

28. 根据权利要求 27 所述的过程, 其中, 所述控制释放制剂进一步包括形成一个或者多个包覆层的步骤, 所述包覆层包括凝胶形成肽。

29. 根据权利要求 27 或 28 所述的过程, 其中, 所述第一自组装凝胶形成肽选自 $\text{Ac}-(\text{RADA})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_5-\text{CONH}_2$ 和 $\text{Ac}-(\text{RADA})_6-\text{CONH}_2$ 。

30. 根据权利要求 27 或 28 所述的过程, 其中, 所述第二凝胶形成肽选自 $\text{Ac}-(\text{RADA})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_4-\text{CONH}_2$ 、 $\text{Ac}-(\text{RADA})_5-\text{CONH}_2$ 和 $\text{Ac}-(\text{RADA})_6-\text{CONH}_2$ 。

31. 根据权利要求 27-28 中任意一项所述的过程, 其中, 所述第一凝胶形成肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。

32. 根据权利要求 17 所述的过程, 其中, 所述第一凝胶形成肽选自 $\text{Ac}-(\text{KLDL})_3-\text{CONH}_2$ 、 $\text{Ac}-(\text{KLDL})_4-\text{CONH}_2$ 和 $\text{Ac}-(\text{KLDL})_5-\text{CONH}_2$ 。

33. 根据权利要求 27-32 中任意一项所述的过程, 其中, 所述治疗剂是蛋白质。

34. 根据权利要求 27-32 中任意一项所述的方法, 其中, 所述治疗剂选自促红细胞生成素 (EPO)、干扰素 - α 、干扰素 - β 、干扰素 - γ 、生长激素、生长激素释放因子、神经生长因子 (NGF)、粒性白细胞 - 集落刺激因子 (G-CSF)、粒性白细胞巨噬细胞 - 集落刺激因子 (GM-CSF)、巨噬细胞 - 集落刺激因子 (M-CSF)、凝血因子、胰岛素、催产素、加压素、促肾上腺皮质激素、表皮生长因子、血小板衍生生长因子 (PDGF)、催乳激素、促黄体素释放素、促黄体素释放激素 (LHRH)、促黄体素释放激素激动剂、促黄体素释放激素拮抗剂、生长激素释放的

抑制因子、胰高血糖素、干扰白细胞素 -2(IL-2)、白介素 -11(IL-11)、促胃液素、四肽胃泌素、五肽促胃酸激素、尿抑胃激素、分泌素、降血钙素、脑啡肽、内啡肽、血管紧张素、促甲状腺素释放激素 (TRH)、肿瘤坏死因子 (TNF)、肿瘤坏死因子有关的凋亡诱导配位体 (TRAIL)、肝素酶、骨形态蛋白 (BMP)、人前房促尿钠排泄肽 (hANP)、胰高血糖素 - 样肽 (GLP-1)、肾素、血管舒缓激肽、杆菌肽、多粘菌素、粘菌素、短杆菌酪肽、短杆菌肽、环孢菌素、小干扰 RNA (siRNA)、质粒 DNA、和反义低聚脱氧核苷酸 (AS-ODN)。

35. 由权利要求 27-34 中任意一项所述的过程制备的持续释放制剂。

用于长时间持续释放人类抗体的多层可注射自组装肽脚手架水凝胶

[0001] 相关申请

[0002] 本申请要求 2013 年 3 月 14 日递交的美国临时申请第 61/782,791 号的权利。上述申请的全部教导通过引证在此全部并入本文。

技术背景

[0003] 由于自 20 世纪 60 年代开始,控制释放主要集中在聚合物水凝胶和动物衍生的生物材料,因此使用水凝胶作为药物传递载体已经被研究过了。但是,由合成聚合物组成的水凝胶由于以下原因不能代表生物医学应用的理想系统:(i) 组分和降解产物的毒性(例如,许多聚合物需要利用有毒的交联剂,例如戊二醛,及其他能够威胁生命的化学试剂,例如聚乙醇酸-聚乳酸及其类似物,在降解过程中局部释放酸),(ii) 后凝胶化聚合物膨胀常常会导致宿主疼痛,和(iii) 由于聚合物网络的大孔,活性聚合物在较短的时间内释放。

[0004] 而且,在实际涉及人体的临床应用中不会考虑从动物体提取的生物聚合物,例如,胶原蛋白、明胶、纤维蛋白和粘连蛋白^[1,2,3,4],这是由于这些生物聚合物的来源和具有引发宿主对可能存在于供体组织中的病毒、细菌及其他未知物质的炎症性反应的风险。为了满足生物相容性药物释放系统的需要,研发了可生物降解的合成聚合物^[5,6,7,8]。尽管对新型水凝胶系统进行了广泛的研究和持续的研发,但是这些挑战没有被完全攻克。

[0005] 之前,研究了由肽 ac-(RADA)₄-CONH₂(其中, R 是精氨酸, A 是丙氨酸并且 D 是天冬氨酸)自组装组成的纳米纤维水凝胶对小模型药物分子的控制释放^[9]。在近期研究中已经表明,具有不同分子量和等电点的蛋白质可以通过 ac-(RADA)₄-CONH₂肽水凝胶缓慢释放,并且研究了在 3 个月时间内的释放动力学^[10]。自组装肽水凝胶是可注射的,这是由于他们可以在体内随着肽溶液和生物液体的相互作用形成。在被引入电解质溶液过程中,自组装肽形成直径在 10 纳米-20 纳米之间的纳米纤维,这进一步组织形成含水率高达 99.5% (重量/体积) 的脚手架水凝胶,并且形成的小孔直径在 5 纳米-200 纳米之间^[11]。肽的凝胶化不需要有害的材料(例如毒性交联剂)来引发溶胶-凝胶变化,并且这种水凝胶的降解产物是天然氨基酸,这种天然氨基酸可以被代谢并被身体重新利用。溶胶-凝胶变化在生理条件下进行的事实促进了肽溶液与生物活性分子的混合,并且以一种组织特异性方式被共同注射,在组织中形成药物传递载体。根据分子设计,肽脚手架水凝胶是生物相容的,并且已经被用于许多组织工程应用,包括骨和软骨再造、神经元的和心脏组织再生、伤口愈合、血管生成和止血^[12,13]。自组装肽水凝胶提供了一种平台,该平台使其可以理想的用于各种生物纳米医学应用,用于促进水凝胶内部的细胞迁移。而且他们是无毒的、非免疫原性的、非凝血酶原性的、生物可降解的,并且适合于通过注射入某一具体组织而进行定位治疗^[14,15]。

[0006] 由于除了存在复杂的三维结构之外还存在多种功能性基团,大蛋白质,例如抗体,比传统的有机药物和无机药物更大并且更为复杂,因此他们的控制释放位点制剂更难攻克。为了使抗体维持生物活性,该制剂必须在整个治疗期间保护抗体的功能性。在持续释

放期间,由于三维结构的缺失或者化学不稳定性,抗体有多种降解途径。这些挑战被治疗长度弱化。具有治疗性质的功能性抗体的呈递对于持续传递生物医学应用是重要的。因此,需要开发一种基于生物可降解的肽的持续释放系统,从而在延长的时间期限内传递治疗有效性大蛋白质,例如,抗体。

发明内容

[0007] 本发明涉及一种用于持续传递治疗剂的药物制剂,所述治疗剂优选蛋白质、多肽、抗体或者抗体片段,所述药物制剂包括一种或者一种以上胶体形成肽,其中,所述制剂在至少 2 周、至少三周、至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周、至少十一周、至少十二周或者更长的时间内显示持续的传递。在一个实施方案中,本发明涉及一种制剂,所述制剂包括在电解质溶液(例如,生物流体和盐)存在的情况下经历溶胶-凝胶变化的自组装肽。所述制剂可以持续释放抗体和抗体片段,尤其是 IgG。随着水凝胶纳米纤维密度的增加,抗体的扩散率会减少,从而提供了一种控制释放动力学的方式。

[0008] 本发明进一步涉及多层水凝胶结构,这些多层水凝胶结构包括基本上同心球状的补体和结构上相容肽,例如 ac-(RADA)₄-CONH₂核心和 ac-(KLDL)₃-CONH₂壳。所述治疗剂可以通过洋葱样的体系结构扩散,当使用补体肽时可以形成所述洋葱样的体系结构。在一个实施方案中,本发明的持续释放制剂可以在至少两周、至少三周、至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周、至少十一周和至少十二周或更长的时间内释放功能上完整的抗体。在一种优选的实施方案中,所述制剂在至少大约 2 个月到大约三个月的时间内持续释放功能上完整的抗体,例如 IgG。

[0009] 在一个实施方案中,本发明涉及一种制剂,其中,被释放的抗体(例如, IgG)的二级结构和三级结构分析以及生物学分析显示,即使在水凝胶内部两个或者三个月之后,密封和释放不影响所述抗体的构象和他们的生物活性。多克隆人 IgG 的功能性可以通过 IgG 密封和释放之后他们与胆碱磷酸抗原的亲和性来确定。所述抗原结合效率可以被用于确定密封后功能性的保留度。本发明涉及一种制剂,其中一种完全生物相容性可注射肽水凝胶系统可以用于控制释放应用,作为治疗剂抗体的载体。

[0010] 在一种优选的实施方案中,本发明涉及人免疫球蛋白(pI 7.1, 分子量 146kDa)的持续释放制剂,在大约 3 个月的时间内包括 -ac-(RADA)₄-CONH₂ 和 ac-(KLDL)₃-CONH₂ 自组装肽水凝胶。在一种优选的实施方案中,使用圆振二向色性(CD)、荧光光谱分析和免疫测定法对释放的抗体进行分析,据此可知,整合肽和从肽水凝胶中释放抗体的过程基本上不影响其构象和功能。可以使用单分子荧光相关光谱(FCS) 和石英晶体微量天平(QCM) 生物传感器技术监控 IgG 的生物活性。

[0011] 本发明进一步涉及一种持续药物传递系统,该系统可以有效的将治疗剂导向具体的组织,其中这种定向传递对病人产生更小的毒性和副作用。所述可注射的自组装肽脚手架系统,其中,在生理条件下,所述凝胶可以被用于包括免疫治疗的持续释放应用,从而在延长的时间内将活性抗体局部释放到具体的组织中。在一种优选的实施方案中,在大约 3 个月的时间内,人类抗体通过 ac-(RADA)₄-CONH₂ 或者 ac-(KLDL)₃-CONH₂ 肽水凝胶缓慢释放。所述释放动力学随着形成水凝胶的自组装肽氨基酸序列的变化和水凝胶中肽纳米纤维的密度变化而改变。而且,所述控制释放系统可以呈递生物活性蛋白质,其中,所述水凝胶释

放的抗体的二级结构和三级结构以及他们的生物活性基本上不受密封和通过水凝胶释放的影响。肽序列的可编程性是独特的，并且提供了一种将纳米纤维性质控制在分子水平的方法，这反过来可以改变生物分子的扩散和释放动力学。

[0012] 附图简要说明

[0013] 本发明的前述及其他目标、特征和优势从下列对本发明优选的实施方案的更为具体的描述中变得显而易见，如随后附图中说明的那样，其中，不同视图中相同的附图标记指的是相同的部分。所述附图不需要按比例绘制，相反，在图示上突出重点说明本发明的原则。

[0014] 图1是(A) ac-(RADA)₄-CONH₂肽单体、和其肽纳米纤维的图示、(B) IgG分子的图示、(c) 肽纳米纤维的电子镜检术图像图示和(D) 脚手架水凝胶的宏观图像图示。IgG 和肽的色彩设计：带阳电荷的（蓝色）、带负电荷的（红色）和疏水性的（灰色）。

[0015] 图2. (A) 多层水凝胶的示意图。(B) 多层水凝胶的光学显微镜检查和(C-D) 多层水凝胶的荧光显微镜检查。为了便于观察，壳（自组装 ac-(KLDL)₃-CONH₂肽）装载有 Alexa-488 荧光基团 (C) 并且核（自组装 ac-(RADA)₄-CONH₂肽）装载有 CY3 (D)。

[0016] 图3. (A) IgG 通过不同肽和不同肽纳米纤维密度水凝胶在整个3个月的时间内的释放曲线 (A) 和在头12天的释放曲线 (B)。由自组装肽 (i) ac-(RADA)₄-CONH₂，浓度分别为0.5%重量/体积的（淡蓝色，▲）、1.0%重量/体积（蓝色，▲）、和1.5%重量/体积（深蓝色，▲）和 (ii) ac-(KLDL)₃-CONH₂，浓度分别为0.3%重量/体积（红色，■）和0.6%重量/体积（品红色，■）。在室温条件下，pH值为7.4，磷酸缓冲液中进行释放试验。数据点表示五个样品的平均计算标准偏差小于12%。(C) 将 IgG 释放曲线绘制成时间平方根的函数，显示双相的扩散机理。绘图最初的线性部分代表 IgG 通过所述肽水凝胶的单向扩散，可以根据菲克定律（等式1）计算扩散系数。

[0017] 图4. IgG 通过水凝胶和多层双组分水凝胶在整个3个月的时间内的释放曲线 (A) 和在头12天的释放曲线 (B)，所述水凝胶由浓度为1.0%重量/体积的 ac-(RADA)₄-CONH₂（蓝色，▲）和浓度为0.6%重量/体积的 ac-(KLDL)₃-CONH₂（品红色，■）所组成，所述多层双组分水凝胶由浓度为1.0%重量/体积的 ac-(RADA)₄-CONH₂（核心）和浓度为0.6%重量/体积的 ac-(KLDL)₃-CONH₂（壳）所组成。在室温条件下，pH值为7.4，磷酸缓冲液中进行释放试验。数据点表示五个样品的平均计算标准偏差小于12%。(C) 将 IgG 释放曲线绘制成时间平方根的函数，显示双相的扩散机理。绘图最初的线性部分代表 IgG 通过所述肽水凝胶的单向扩散，可以根据菲克定律（等式1）计算扩散系数。

[0018] 图5. 人 IgG 在 pH 值为 7.4 的磷酸缓冲液中的光谱分析 (A) 天然 IgG(实线) 和肽水凝胶释放的 IgG(虚线) 的远紫外线圆二色性谱。(B) 天然 IgG(实线) 和通过肽水凝胶释放的 IgG(虚线) 的标准化的荧光发射光谱；激发波长是 300 纳米。释放样品后 2 个月的时间内在室温下记录光谱。

[0019] 图6. 游离的 IgG 分子在不存在和存在 PC-BSA 抗原情况下，在通过 ac-(RADA)₄-CONH₂肽水凝胶释放之前和之后的平移扩散时间。单分子荧光相关光谱 (FCS) 数据显示 IgG 与抗原的相互作用产生三种不同的种类：游离的 IgG 分子，其扩散时间与单独的 IgG 扩散时间相似，和与一个或者两个抗原分子键合的 IgG。在 IgG 释放前后这些种类的相对浓度是相似的。标准偏差在 5% 和 12% 之间。

[0020] 图 7. 天然多克隆人 IgG(A) 和 (B) 水凝胶释放的多克隆人 IgG 与固定的 PC-BSA(抗原) 相连 (结合) 和解离的 QCM 随时间而变的频率变化, $-dF$ 。零时代表在流体管道中注入 IgG 的时间, 随后是结合相。随着 IgG 的注射和 IgG 与固定抗原的结合, 生物传感器信号 (黑线) 增加, 随着缓冲液的注射 (箭头) 导致结合的 IgG 解离, 生物传感器信号降低。天然 IgG 和水凝胶释放的 IgG 之间计算的速率常数的相似性和亲合常数的相似性说明 IgG 的功能特点在整合和通过肽水凝胶释放的过程中不受影响。红线代表数据拟合。

[0021] 图 8. 使用史托 - 爱因斯坦方程确定人免疫球蛋白 (IgG, 分子量 146kDa, 流体动力学半径 $r_h = 5.3$ 纳米, 等电位点 pI 7.1) 在溶液中的扩散常数, 并随着通过肽水凝胶释放过程使用菲克定律确定扩散常数。使用误差传递计算标准偏差 ($n = 8$)。

具体实施方式

[0022] 本发明涉及一种用于持续传递抗体或者抗体片段的药物制剂, 所述抗体或者抗体片段包括一种或者一种以上胶体形成肽, 其中, 所述制剂在至少 2 周、三周、四周、五周、六周、七周、八周、九周、十周、十一周、十二周或者更长的时间内显示持续的传递。在一个实施方案中, 本发明涉及一种制剂, 所述制剂包括在电解质溶液 (例如, 生物流体和盐) 存在的情况下经历溶胶 - 凝胶变化的自组装肽。所述制剂可以持续释放抗体和抗体片段, 尤其是 IgG。随着水凝胶纳米纤维密度的增加, 抗体的扩散率会减少, 从而提供了一种控制释放动力学的方式。

[0023] 本发明进一步涉及多层水凝胶结构, 这些多层水凝胶结构包括基本上同心球状的补体和结构上相容肽, 例如 ac-(RADA)₄-CONH₂核心和 ac-(KLDL)₃-CONH₂壳。所述抗体可以通过洋葱样的体系结构扩散, 当使用补体肽时可以形成所述洋葱样的体系结构。本发明的持续释放制剂可以在至少两周、至少三周、至少四周、至少五周、至少六周、至少七周、至少八周、至少九周、至少十周、至少十一周和至少十二周或更长的时间内释放功能上完整的抗体。在一种优选的实施方案中, 所述制剂在至少大约 2 个月到大约三个月的时间内持续释放功能上完整的抗体, 例如 IgG。

[0024] 本发明进一步涉及一种制备多层凝胶制剂的方法, 其中, 形成包括治疗剂的第一核心凝胶, 并且进一步形成另一层凝胶包裹密封所述核心凝胶。核心凝胶的形成可以随着凝胶形成肽在适当的水溶液中的混合而发生, 并且等待凝胶形成。在一个实施方案中, 向缓冲溶液中加入凝胶形成肽并混合。在一个实施方案中, 通过在注射器针头的顶端形成液滴并允许发生凝胶化作用来形成滴状凝胶。

[0025] 在一个实施方案中, 本发明涉及一种制剂, 其中, 被释放的抗体 (例如, IgG) 的二级结构和三级结构分析以及生物学分析显示, 即使在水凝胶内部两个或者三个月之后, 密封和释放不影响所述抗体的构象和他们的生物活性。多克隆人 IgG 的功能性可以通过 IgG 密封和释放之后他们与胆碱磷酸抗原的亲和性来确定。所述抗原结合效率可以被用于确定密封后功能性的保留度。本发明涉及一种制剂, 其中一种完全生物相容性可注射肽水凝胶系统可以用于控制释放应用, 作为治疗剂抗体的载体。

[0026] 在三个月内研究人免疫球蛋白 (IgG) 通过纳米纤维脚手架水凝胶的释放动力学, 所述纳米纤维脚手架水凝胶由 ac-(RADA)₄-CONH₂ 和 ac-(KLDL)₃-CONH₂ 自组装肽组成。自组装肽是一类刺激响应性材料, 其在电解质溶液 (例如, 生物流体和盐) 存在的情

况下经历溶胶-凝胶变化。随着水凝胶纳米纤维密度的增加, IgG 的扩散率会减少, 从而提供了一种控制释放动力学的方式。多层水凝胶结构还可以由 ac-(RADA)4-CONH2 核心和 ac-(KLDL)3-CONH2 壳组成的同心球构成。. 确定所述抗体通过洋葱样结构的扩散曲线。释放的 IgG 的二级结构和三级结构分析以及生物学测定显示即使在水凝胶内部存在 3 个月之后, 密封和释放不会影响所述抗体的构象及其生物活性。确定多克隆人 IgG 对胆碱磷酸抗原的功能性, 结果显示 IgG 密封和释放不影响所述抗体与抗原的结合效力。我们的结果显示这种完全生物相容性可注射肽水凝胶系统可以用于控制释放应用, 作为治疗剂抗体的载体。

[0027] 本发明进一步涉及一种持续药物传递系统, 该系统可以有效的将治疗剂导向具体的组织, 其中这种定向传递对病人产生更小的毒性和副作用。所述可注射的自组装肽脚手架系统, 其中, 在生理条件下, 所述凝胶可以被用于包括免疫治疗的持续释放应用, 从而在延长的时间内将活性抗体局部释放到具体的组织中。在一种优选的实施方案中, 在大约 3 个月的时间内, 人类抗体通过 ac-(RADA)4-CONH2 或者 ac-(KLDL)3-CONH2 肽水凝胶缓慢释放。所述释放动力学随着形成水凝胶的自组装肽氨基酸序列的变化和水凝胶中肽纳米纤维的密度变化而改变。而且, 所述控制释放系统可以呈递生物活性蛋白质, 其中, 所述水凝胶释放的抗体的二级结构和三级结构以及他们的生物活性基本上不受密封和通过水凝胶释放的影响。肽序列的可编程性是独特的, 并且提供了一种将纳米纤维性质控制在分子水平的方法, 这反过来可以改变生物分子的扩散和释放动力学。

[0028] 在一个实施方案中, 本发明涉及包括第一肽单体和一种抗体的核心, 其中, 所述核心被第二肽层包覆。在一种优选的实施方案中, 所述核心包括第一肽, 所述第一肽选自 Ac-(RADA)3-CONH2、Ac-(RADA)4-CONH2、Ac-(RADA)5-CONH2; 并且所述肽选自 Ac-(KLDL)3-CONH2、Ac-(KLDL)4-CONH2、Ac-(KLDL)5-CONH2; 并且所述抗体是 IgG 抗体。所述多层水凝胶结构可以通过两步凝胶化过程制备。例如, 随着 ac-(RADA)4-CONH2 肽溶液 (大约 0.2 到大约 5.0% 重量 / 体积; 优选在大约 0.4 到大约 2.0% 重量 / 体积之间; 优选在大约 0.5 到大约 1.5% 重量 / 体积之间) 与包含 IgG (大约 1 微摩到大约 20 微摩; 优选在大约 2 微摩到大约 10 微摩之间; 4 微摩到大约 8 微摩之间) 的缓冲溶液 (磷酸缓冲液或者其他适用凝胶形成的缓冲液) 的混合, 可以形成包含 IgG 的凝胶。所述凝胶可以形成理想的物理形状。例如, 使用注射器在针头尖端形成液滴, 并等待足够的时间允许凝胶化, 形成基本上球形的核心结构。自组装过程产生具有基本上球面几何形状的水凝胶, 附着在注射器针头的尖端。形成的第一凝胶可以被第二层自组装肽包覆, 产生分层的产物。例如, 使用第二注射器, 另一个自组装肽层 ac-(KLDL)3-CONH2 可以被用于所述核心的外表面。所述 ac-(KLDL)3-CONH2 肽溶液可以是大约 0.1 到大约 5.0% 重量 / 体积; 优选的在大约 0.2 到大约 2.0% 重量 / 体积之间; 优选的, 在大约 0.3 到大约 1.5% 重量 / 体积之间; 或者更为优选的是大约 0.6% 重量 / 体积。在最后一步, 发生凝胶化作用之前, 将一滴 ac-(KLDL)3-CONH2 肽溶液与预先形成的 ac-(RADA)4-CONH2 肽水凝胶相接触并包覆密封之前形成的 ac-(RADA)4-CONH2 肽水凝胶, 从而产生洋葱样的多层水凝胶结构。上述步骤形成具有 IgG 抗体和自组装肽的核心, 随后被第二肽包覆, 这一过程可以重复多次从而得到多层洋葱样结构。例如, 上面描述的产物包含包覆有 ac-(KLDL)3-CONH2 的 ac-(RADA)4CONH2/IgG 核心, 并且根据所需的释放曲线, 上述产物可以进一步包覆一层 ac-(RADA)4CONH2/

IgG, 随后在包覆另一层 ac-(KLDL) 3-CONH2。

[0029] 其他自组装肽还可以用于形成一个或者一个以上层或者所述制剂的核心。这些肽的实施例包括:K27, 这是一种二十七个氨基酸的 β 片型肽 (KLEALYIIMVLGFFGFFILGIMLSYIR) ;K24, 这是一种二十四个氨基酸的 β 片型肽 (KLEALYVLGFFGFFILGIMLSYIR) ;或者 Boden et al. (美国专利公开号 20020132974) 描述的 SER-1 ;或者 Lockwood et al. (Biomacromolecules, 2002, 3 (6), pp1225-1232) 中描述的 PF424_46。

[0030] 其他胶体形成剂或者包覆试剂 (例如透明质酸、胶原蛋白、交联聚合物、氨基多糖、角蛋白、粘连蛋白、玻璃体结合蛋白、或者粘连蛋白) 可用于形成包裹密封这里所描述制剂核心的一个或者一个以上层。

[0031] 本发明涉及包含一个或者一个以上治疗剂的制剂。在一种优选的实施方案中, 所述治疗剂选自抗体、IgG 抗体、单克隆抗体、多克隆抗体、单克隆抗体片段和多克隆片段, 其中所述治疗剂在至少大约 1 个月、或者 2 个月或者 3 个月的时间内释放。

[0032] 术语“抗体”从广义上讲, 具体的说, 包括单克隆抗体 (包括全长单克隆抗体)、多克隆抗体、多特异性抗体 (例如, 双特异性抗体) 和抗体片段, 只要他们显示出所需的生物活性。

[0033] “抗体片段”包括全长抗体的一部分, 通常指其抗原结合区域或者可变区域。抗体片段的实施例包括 Fab、Fab'、F(ab')₂ 和 Fv 片段; 双体; 线性抗体; 单链抗体分子; 和由抗体片段形成的多特异性抗体。

[0034] 如这里所使用的术语“单克隆抗体”指的是从基本上同族的抗体群中获得的抗体, 即, 所述群中各个单独的抗体除了其中少数可能包括一些天然存在的突变之外都是相同的。单克隆抗体是高度特异性的, 导向单个抗原位点。传统 (多克隆) 抗体制剂一般包括针对不同决定簇 (抗原决定簇) 的不同抗体, 与此不同, 每个单克隆抗体针对抗原上的单个决定簇。调节剂“单克隆”指的是从基本上同族抗体中获得的抗体特性, 并且不被看成通过任何特定的方法生产抗体。例如, 根据本发明, 这里使用的单克隆抗体可以通过如 Kohler et al, Nature 256 :495 (1975) 中首先描述的杂交方法制备, 或者通过重组 DNA 的方法制备 (参见, 例如, 美国专利第 4,816,567 号)。所述“单克隆抗体”还可以从噬菌体抗体文库中使用例如 Clackson et al., Nature 352 :624-626 (1991) 和 Marks et al., J. Mol. Biol. 222 :581-597 (1991) 中描述的技术分离出来。.

[0035] 在一种优选的实施方案中, 抗体选自免疫球蛋白 IgD、免疫球蛋白 IgA、免疫球蛋白 IgM、免疫球蛋白 IgE 或者免疫球蛋白 IgG。在一个更优选的实施方案中, 所述抗体是 IgG。在一种优选的实施方案中, 所述抗体选自由 IgG1、IgG2、IgG3 和 IgG4、IgM1 和 IgM2、和 IgA1 和 IgA2 抗体所组成的组中。在一个实施方案中, 所述抗体是全抗体或者单链 Fv 抗体片段或者 Fab 抗体片段。在一种优选的实施方案中, 所述抗体选自美罗华、英夫利昔单抗、曲妥珠单抗、阿昔单抗、帕利珠单抗、Murumonab-CD3、吉妥单抗、曲妥珠单抗、巴利昔单抗、达(克)珠单抗、依那西普和替伊莫单抗、伊莫单抗。在一个实施方案中, 所述抗体选自抗肿瘤坏死因子抗体、抗-CD3 抗体、抗-CD20 抗体、抗-CD25 抗体、抗-CD33 抗体、抗-CD40 抗体、抗-HER2 抗体、抗-HBV 抗体、抗 HAV 抗体、抗 HCV 抗体、抗 GPIIb/IIIa 受体抗体、抗 RSV 抗体、抗 HIV 抗体、抗 HSV 抗体和抗 EBV 抗体。

[0036] 非人（例如，鼠）抗体的人源化形式是嵌合抗体，其包含来源于非人免疫球蛋白的最小序列。在很大程度上，人源化抗体是人免疫球蛋白（受体抗体），其中从受体高可变区中获得的残基可以被来自非人种族（供体抗体）高可变区中获得的残基所取代，其中，所述非人种族是例如，小鼠、大鼠、兔或者其他具有所需专一性、亲和性和容量的非人灵长类动物。在有些情况下，人免疫球蛋白的骨架区残基可以被相应的非人残基所取代。而且，人源化抗体可以包括受体抗体或者供体抗体中没有发现的残基。进行这些修饰从而进一步改善抗体性能。通常，所述人源化抗体基本上包括至少一个、通常是两个可变域，其中，所有或者基本上所有高可变区对应于非人免疫球蛋白的可变域，并且，所有或者基本上所有骨架区是人免疫球蛋白序列的可变域。选择性的，所述人源化抗体还包括至少免疫球蛋白恒定区（Fc）的至少一部分，通常是人免疫球蛋白恒定区的至少一部分。为了了解详情，参见 Jones et al., Nature 321 :522-525 (1986) ;Riechmann et al, Nature332 :323-329 (1988) ;和 Presta, Curr. Op. Struct. Biol. 2 :593-596 (1992).

[0037] “单链 Fv”或者“sFv”抗体片段包括抗体的重链可变区（VH）和轻链可变（VL）结构域，其中这些结构域存在于单个多肽链中。通常，所述 Fv 多肽在重链可变区（VH）和轻链可变（VL）结构域之间进一步包括一种多肽连接体，这能够使 sFv 形成所需的结构用于结合抗原。参见 sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 1 13, Rosenburg 和 Moore eds. Springer-Verlag, N. Y. , pp. 269-315 (1994).

[0038] IgG 是血液中抗体最丰富的种类（血清浓度是 13 毫克 / 毫升）。IgG 有四个亚类，这四类全部是单体并且他们通常对抗原具有非常高的亲合性。产生的抗体 IgG 的亚类取决于其呈递的细胞因子（特别是 IL-4 和 IL-2），并且每个亚类有其独有的特殊活性。IgG 还在中和血液和组织中毒素（例如来自细菌感染）方面发挥重要作用。

[0039] 例如，在抗原结合实验中确定，如果在给定时间内抗体生物活性与药物制剂被制备时所显示的生物活性相比，变化在大约 20%（在实验误差范围内）范围内，则认为药物制剂中的抗体“保持其生物活性”。

[0040] 多种治疗剂，例如，蛋白质、多肽、微 RNA 及其他生物聚合物也可以被整合到所述制剂中进行持续释放。在一个实施方案中，所述治疗剂选自促红细胞生成素（EPO）、干扰素 - α 、干扰素 - β 、干扰素 - γ 、生长激素、生长激素释放因子、神经生长因子（NGF）、粒性白细胞 - 集落刺激因子（G-CSF）、粒性白细胞巨噬细胞 - 集落刺激因子（GM-CSF）、巨噬细胞 - 集落刺激因子（M-CSF）、凝血因子、胰岛素、催产素、加压素、促肾上腺皮质激素、表皮生长因子、血小板衍生生长因子（PDGF）、催乳激素、促黄体素释放素、促黄体素释放激素（LHRH）、促黄体素释放激素激动剂、促黄体素释放激素拮抗剂、生长激素释放的抑制因子、胰高血糖素、干扰白细胞素 -2 (IL-2)、白介素 -11 (IL-11)、促胃液素、四肽胃泌素、五肽促胃酸激素、尿抑胃激素、分泌素、降血钙素、脑啡肽、内啡肽、血管紧张素、促甲状腺素释放激素（TRH）、肿瘤坏死因子（TNF）、肿瘤坏死因子有关的凋亡诱导配体（TRAIL）、肝素酶、骨形态蛋白（BMP）、人前房促尿钠排泄肽（hANP）、胰高血糖素 - 样肽（GLP-1）、肾素、血管舒缓激肽、杆菌肽、多粘菌素、粘菌素、短杆菌肽、短杆菌肽、环孢菌素、小干扰 RNA（siRNA）、质粒 DNA、和反义低聚脱氧核苷酸（AS-ODN）。

[0041] 用作抗粉刺试剂、抗过敏试剂、抗焦虑药、平喘药、抗癌剂、降血脂剂、激素避孕药、抗抑郁药、抗糖尿病药、镇痛剂、抗重症肌无力药、抗高血压剂、抗真菌剂、抗生素、助眠药、

激素治疗剂、抗偏头痛药、用于治疗超重的药物、抗帕金森病试剂、抗精神病药、非类固醇抗炎性药物、排卵诱导剂、粘液溶解剂、止咳药、勃起诱导剂和抗溃疡药的治疗剂可以被整合到这里所描述制剂的核心结构中,用于持续传递。在一种优选的实施方案中,本发明涉及制剂,所述制剂包括选自胰岛素、亮脯利特、促性腺激素释放因子、生长激素释放的抑制因子、奥曲肽、恩夫韦肽、降血钙素和奈西立肽所组成的组中的治疗剂。

[0042] 在另一个实施方案中,所述治疗剂是一种血栓溶解剂,选自安克洛酶、复合纤溶酶链激酶、甲氧丁喹乳酸盐、米曲溶纤维蛋白酶、哈格曼因子(即,因子XII)片段、吗斯酮胺、纤维蛋白溶酶原活化因子例如链激酶、组织纤溶酶原激活物(TPA)、尿激酶、TFPI、血纤维蛋白溶酶和血纤维蛋白溶酶原,以及凝血因子IIa、Va、Vila、Villa、IXa、Xa、XIa、XIIa和XIIa的抑制剂。

[0043] 在另一个实施方案中,所述治疗剂是一种细胞信号传导分子,选自:细胞因子、生长因子、白介素、 α -干扰素、 γ -干扰素、转化生长因子- β 、嗜中性白细胞活化肽-2及其类似物、巨噬细胞炎症性蛋白质及其类似物、和血小板-因子4和转化生长因子- β 及其组合。

[0044] 在另一个实施方案中,所述治疗剂是消炎剂,选自:阿氯芬酸、阿氯米松二丙酸盐、丙缩阿尔孕酮、 α 淀粉酶、安西法尔、安西非特、氨芬酸钠、氨普立糖盐酸盐、阿那白滞素、阿尼罗酸、阿尼扎芬、炎爽痛、阿斯匹林、巴柳氮二钠、苄达酸、苯恶洛芬、盐酸炎痛静、菠萝蛋白酶、溴四唑哌啶、布地缩松、卡洛芬、芴丙酸、噌戊唑酮、克利洛芬、丙酸氯氟美松、氯氟美松酮丁酸盐、氯吡酸、氯硫卡松丙酸盐、可米松醋酸盐、11-去氧可的松、地夫可特、地索奈德、去羟米松、地塞米松二丙酸盐、双氯芬酸钾、双氯灭痛、双醋二氟拉松、二氟米酮钠、二氟苯水杨酸、二氟泼尼酯、地弗酞酮、二甲亚砜、羟西奈德、蜕皮激素、恩莫单抗、依诺利康钠、甲嘧啶唑、依托度酸、依托芬那酯、联苯乙酸、非那莫、联苯丁酮酸、芬氯酸、苯克洛酸、苯吲柳酸、苯吡恶二酮、双苯噻酸、夫拉扎酮、氟恶米松、氟灭酸、氟咪唑、氟尼缩松醋酸盐、氟胺烟酸、氟胺烟酸甲基葡胺、氟考丁酯、氟甲脱氧泼尼松龙醋酸盐、氟喹宗、氟比洛芬、氟瑞托芬、氟替卡松丙酸盐、呋喃洛芬、呋罗布芬、哈西缩松、卤贝他索丙酸盐、卤泼尼松醋酸盐、对异丁基苯乙酸、布洛芬、布洛芬铝、布洛芬吡啶甲醇、伊洛达普、吲哚氯甲酰、吲哚氯甲酰钠、吲哚布洛芬、双甲氧苯吲哚、吲四唑、异氟泼尼龙醋酸盐、伊索克酸、异恶噻酰胺、苯酮苯丙酸、洛非咪唑盐酸盐、氯诺昔康、氯替泼诺、甲氯灭酸钠、甲氯灭酸、二丁酸甲氯松、甲灭酸、马沙拉嗪、美西拉宗、甲基氯化泼尼松磺庚酯、莫尼氟酯、萘布美酮、萘普生、萘普生钠、萘普索、尼马宗、奥撒拉嗪钠、肝蛋白、奥帕诺辛、奥沙普秦、羟保泰松、瑞尼托林盐酸盐、戊聚糖聚硫酸钠、保泰松甘油酸钠、吡非尼酮、吡罗昔康、吡罗昔康肉桂酸盐、吡罗昔康胆胺、吡丙芬、泼那扎特、普立非酮、普罗度酸、普罗喹宗、普罗沙唑、普罗沙唑柠檬酸盐、双甲丙酰龙、氯马扎利、柳胆来司、沙那西定、双水杨酯、水杨酸盐、氯化鸡志贺氏、司克拉宗、丝美辛、舒多昔康、舒林酸、舒洛芬、他美辛、他尼氟酯、醋柳酰酯、替布费龙、替尼达普、替尼达普钠、替诺昔康、替昔康、苄叉异喹酮、毒鼠强、二氢氧二苯并硫杂、替可的松新戊酸酯、甲苯酰吡啶乙酸、甲苯酰吡啶乙酸钠、三氯氟松、三氟氨酯、叠氮吲酸、糖皮质激素和佐美酸钠。

[0045] 在另一个实施方案中,所述治疗剂是ADP受体拮抗体,选自噻氯匹定、氯吡格雷、普拉格雷、苯磺唑酮、AZD6140、AZD6933和AR-C69931;糖蛋白I1b/IIIa受体抑制物,选自:阿昔单抗、夫雷非班、拉米非班、洛曲非班、奥波非班、罗昔非班、西拉非班、和替罗非班。

[0046] 在另一个实施方案中,所述治疗剂是抗凝血剂,选自:维生素K拮抗剂、香豆素和香豆素衍生物、华法令钠;肝素;达肝素钠;比伐卢定;溴茚酮;香豆素达特肝素钠;地西卢定;双香豆素;聚烯磺酸钠;萘莫司他甲磺酸盐;苯丙香豆醇脑硫脂;和亭扎肝素钠。

[0047] 在另一个实施方案中,所述治疗剂是脂质还原剂,选自:苯扎贝特、环丙贝特、安妥明、依托贝特、非诺贝特、二甲苯氧庚酸、氯烟贝特和安妥明丙二醇酯;斯达汀、阿伐他汀、西立伐他汀、氟伐他汀、洛弗斯特丁、美伐他汀、匹伐他汀、普伐他汀、罗苏伐他汀和辛伐他汀;烟酸或者烟酸衍生物,包括阿西莫司、烟酸铝、烟酸戊四醇酯(戊赤藓醇四烟酸酯)、烟酰糖酯和烟醇;胆汁酸多价螯合剂,包括考来维仑、考来烯胺、降脂树脂II号和考来糖酐;CETP抑制剂,例如安塞曲匹;苯氟雷司;消胆胺;右旋甲状腺素;依泽替米贝;拉罗匹仑;美格鲁托;ω-3-三酸甘油酯;甘蔗脂肪醇;普罗布考和羟硫癸烷。

[0048] 在另一个实施方案中,所述治疗剂是环加氧酶-2(COX-2)抑制剂,选自阿斯匹林、塞来考昔、罗美昔布和依托考昔。

[0049] 在另一个实施方案中,所述治疗剂是血管紧张素系统抑制剂,选自血管紧张肽转化酶(ACE)抑制剂、血管紧张素II受体拮抗体、能够活化血管紧张素II分解代谢的试剂和能够预防血管紧张素II最终衍生的血管紧张素I合成的试剂;ACE抑制剂选自:苯那普利、卡托普利、西拉普利,地拉普利,依那普利,赖诺普利、福辛普利,咪达普利,培哚普利,莫昔普利、喹那普利、雷米普利、螺普利,替莫普利,群多普利与佐芬普利;血管紧张素II受体拮抗体选自:阿齐沙坦,坎地沙坦,依普沙坦,厄贝沙坦,氯沙坦,奥美沙坦、他索沙坦、替米沙坦、和缬沙坦;一种化疗抗癌药物选自:白消安、亚硝脲氮芥、苯丁酸氮芥、环磷酰胺、阿霉素、雌氮芥、hepsulfan、羟基尿素、异环磷酰胺、环己亚硝脲、苯丙氨酸氮芥、氨甲蝶呤、双溴丙基哌嗪和硫替派。

[0050] 在另一个实施方案中,所述治疗剂是凝栓质降解物合成酶抑制剂,选自阿斯匹林、β-[4-(2-羧基-丙烯基)苯甲基]盐酸吡啶(OKY-1555)和1-羧基庚基和1-羧基-辛基-咪唑、4(Z)-6-[(4RS,5SR)-4-(2-羟苯基)-1,3-二恶烷-5-基]-己-4-烯酸、BM-573、卡莫格雷、CGS-12970、达曲班、达美格雷、DTTX30、E-6700、FCE-27262、咪曲司特(CS-518)、伊波格雷(CV-4151)、酮康唑、KK-505、KY-063、那法格雷(DP-1904)、奥扎格雷(OKY-046)、吡考他胺、吡吗格雷(CGS-13080)、利多瑞尔、SQ29548、罗拉格雷(FCE-22178)、沙替格雷(E-5510)、磺曲苯、特波格雷和UK 38485。

[0051] 在另一个实施方案中,所述治疗剂是JAK-2抑制剂,选自AT9283、VX-680、MK0457、TG101209、ICB018424、LS104、XL019、TG101348、伏立诺他、4-芳基-2-氨基吡啶和4-芳基-2-氨基烷-吡啶。

[0052] 治疗剂的“治疗有效量”指的是在使用抗体进行有效治疗过程中,能够有效预防或者治疗疾病或者异常的量。“疾病或者异常”是指任意能够从所述抗体治疗中获益的情况。这包括慢性异常和急性异常或者疾病,包括是所述哺乳动物易感染所讨论的异常的病理学的情况。

[0053] 呈递在氨基末端冠有乙酰基的肽序列。但是,也可以使用不含有氨基末端冠的肽。合成在C-末端具有氨基甲酰基团的肽序列。然而,还可以使用C末端有羧酸的肽。

[0054] 实施例

[0055] 材料和方法

[0056] 化学品和试剂。从 CPC Scientific (Sunnyvale, 加州, 美国) 处获得粉末态的 ac-(RADA)4-CONH2 和 ac-(KLDL)3-CONH2 肽, 并在麻省理工学院生物聚合物实验室 (剑桥, 麻省, 美国) 定性。从 Sigma-Aldrich 公司 (圣路易斯, 美国密苏里州) 购买 人多克隆 IgG。通过在 PhastSystem 中进行等电点聚丙烯酰胺凝胶电泳确定人 IgG 的 pi 7.1, 使用 IPG 条和蛋白质标准品 (Bio-Rad 实验室, Hercules, CA) 和 IPGphor 系统 (Amersham Pharmacia 生物技术公司, 乌普萨拉, 瑞典)。

[0057] IgG 释放实验。使用已经公知的方法制备肽水凝胶^[9,10,11,12,13]。简要的说, ac-(RADA)4-CONH2 和 ac-(KLDL)3-CONH2 肽溶于去离子水, 并在使用前用探针式超声波仪超声处理 30 分。肽水溶液与磷酸缓冲液 (PBS, pH = 7.4) 相混合, 所述磷酸缓冲液中包含最终浓度为 5 微摩的 IgG。将 40 微升的混合物转入 200 微升聚丙烯管中, 并进行大约 10 分钟的凝胶化作用。随后, 向 40 微升的水凝胶中缓慢加入 70 微升的磷酸缓冲液。为了满足理想的沉没情况并为了确定蛋白质释放曲线, 在较短的时间点将 40 微升的上清液替换为等量的新鲜磷酸缓冲液。在测量过程中, 水凝胶体积不变, 因此, IgG 释放不是水凝胶降解或者膨胀的结果。实验显示聚丙烯管表面上的 IgG 不存在任何可检测的吸附。

[0058] 通过两步凝胶化过程形成多层水凝胶结构。随着 ac-(RADA)4-CONH2 肽溶液 (1.0% 重量 / 体积) 与包含 5 微摩 IgG 的磷酸缓冲液相混合, 我们使用注射器在针的顶端形成液滴, 并等待 10 分钟进行凝胶化作用。自组装过程产生具有基本上球面几何形状的水凝胶, 附着在注射器针头的尖端。然后, 使用不同的注射器将 ac-(KLDL)3-CONH2 肽水溶液与磷酸缓冲液相混合, 所得溶液的最终肽浓度为 0.6% 重量 / 体积。在最后一步, 发生凝胶化作用之前, 将一滴 ac-(KLDL)3-CONH2 肽溶液与预先形成的 ac-(RADA)4-CONH2 肽水凝胶相接触并包覆密封之前形成的 ac-(RADA)4-CONH2 肽水凝胶, 从而产生洋葱样的多层水凝胶结构 (图 2)。将所得的多层水凝胶放置于聚丙烯管底部, 按照之前段落中描述的方法进行扩散实验 (图 2A)。

[0059] 在室温条件下进行 IgG 通过自组装肽纳米纤维脚手架水凝胶的释放实验。使用分光光度计在 280 纳米 (NanoDrop ND-1000UV-Vis, NanoDrop 技术公司, 特拉华, 美国) 下测量上清液中释放的 IgG 的浓度。所有数据点代表了 4 个样品的平均值。使用常见误差传播技术估计计算参数中的不确定性, 即, 对于函数 $y = g(x_1, x_2)$ 使用等式^{9,10,11,12,13} 确定计算值中的误差。

[0060] 显微观察所述多层、双组分水凝胶系统。在观察多层水凝胶之前, 将 ac-(KLDL)3-CONH2 和 the ac-(RADA)4-CONH2 肽溶液分别与 Alexa-488 (绿色) 和 CY3 (红色) 荧光染料溶液相混合。所述多层水凝胶由两个同心球组成, 其含有组合物 1.0% 重量 / 体积 ac-(RADA)4-CONH2 (核心) 和 0.6% 重量 / 体积的 ac-(KLDL)3-CONH2 (壳)。用荧光显微镜 (装备 Hamamatsu 照相机和 Openlab 图象获取软件的尼康 TE300) 进行观察。

[0061] 释放的 IgG 浓度的扩散率测定。对于包括可分子分散的扩散剂的水凝胶基质, 可以使用 Fick 第二扩散定律的 1-D- 非稳定状态形式计算表观扩散系数, 其中, ^[21,22] 给出了时间 (t) 的小值 :

[0062]

$$\frac{M_t}{M_\infty} = \left(\frac{16 D_{app} t}{\pi H^2} \right)^{1/2} \quad (1)$$

[0063] 其中, D_{app} 是表观扩散率, M_t 和 M_∞ 分别是在 t 时和无限时间 (∞) 从水凝胶中释放的扩散化合物的累积量。从肽 - IgG 溶液体积 (即, 40 微升) 和聚丙烯管小孔的直径中计算小孔内水凝胶基质 (H) 的厚度。 D_{app} 可以从数据 M_t/M_∞ vs. $t^{1/2}$ 的线性拟合斜率中获得, 其中 $0 < M_t/M_\infty < 0.6$ 。

[0064] 圆二色性 (CD)。在室温下, 在 190nm-260nm 处记录远 - 紫外圆二色性谱 (Aviv 62DS 分光光度计)。在相同的浓度下将水凝胶释放的 IgG 的圆二色性谱与新制备的 IgG 溶液的圆二色性谱相比较。记录光谱并在 2 秒后进行平均。在 1-mm 石英比色杯中 在 pH 为 7.4 的磷酸缓冲液中进行测量。光谱结果是 4 次扫描的累加和平均。在相同条件下获得不含有 IgG 的缓冲液的空白光谱, 进行减法。

[0065] 荧光发射光谱。使用 Perkin-Elmer LS-50B 分光光度计在室温下, 使用 1 厘米路径的石英比色杯测量释放的 IgG 和天然 IgG 的荧光广度。在 10 纳米 - 400 纳米之间记录发射光谱, 在 300 纳米处激发。分别将激发和发射缝隙宽度设定为 5.0 纳米和 2.5 纳米。样品情况与进行圆二色性测量法时描述的样品情况相同。

[0066] IgG 的 FCS 功能性试验。在室温下, 使用 Fluoropoint 单分子检测系统 (奥林巴斯公司, 东京, 日本) 进行所述抗体 - 抗原结合 FCS 特性鉴定。使用我们所使用的单分子分析系统进行检测时, 蛋白质的荧光信号较小, 因此, 我们用标准蛋白质标记试剂盒 (Molecular Probes 公司, 尤金, OR), 用强荧光团 Alexa-647 标记人 IgG。Alexa-647 荧光标记的 IgG 分子越过共焦的毫微微升体积, 在 633 纳米下被氦氖激光器激发, 测量 (在单位时间内计数) 并使用各自荧光标记的 IgG 在不同的浓度下的校准曲线将测量值转化为浓度。

[0067] 记录共焦体积内荧光强度的变化作为时间的函数和自相关函数, $g(t)$, 这回受到荧光分子的性质以及局部环境下的扩散动力的影响 [16, 17]。为了定义溶液中单分散颗粒的不规则三维扩散的自相关函数 [16, 18, 19], Fluoropoint 系统使用等式 2 :

[0068]

$$g(t) = \frac{1}{N} \left(\frac{1 - F_{trip} + F_{trip} e^{-t/\tau_{trip}}}{1 + (\tau_p/\tau_{trip})} \right) \left(\frac{1}{1 + (\tau_p/\tau_{trip})} \right)^{1/2} \quad (2)$$

[0069] 其中, $g(t)$ 是分数群 (F_{trip}) 和三重态衰变时间 (τ_{trip}) 的函数, N 是样品体内分子的数目, τ_p 是平移扩散时间, s 是一个因子, 描述了圆柱型检测体积, 其等于圆柱底面半径 (ω_0) 除以其高度的一半 (ω_1) 的比率。在完全各向异性溶液中, 扩散分子显著地小于共焦体积, 分子 (例如, IgG) 的扩散系数 D 等于 $D = \omega_0^2/4 \tau_p$, 使用单个和多次平移扩散时间拟合自相关曲线。所有的数据点代表 4 或 8 个样品的平均值。

[0070] 在室温下将 Alexa-647 标记的 IgG 与 10 倍过量的 PC-BSA 抗原 (Athera 生物技术 AB, 瑞典) 在磷酸缓冲液中相互反应 1 小时。IgG 和抗原之间的任何相互作用都会增加复合物的分子量 (和大小), 其特征在于与游离 IgG 相比平移扩散时间 (τ_p) 变慢。通过在不同浓度的吐温清洁剂存在的情况下测量结合亲和性, 从而评价 IgG 与抗原非特异性结合的作用; 在 0.5% 吐温中, 非特异性结合最小, 因此, 所有的 IgG- 抗原结合研究都在此吐温浓度下进行。使用如上所述的 FCS 算法分析数据, 并且使用一个和多次组分拟合自相关函数。

[0071] IgG 的 QCM 功能性试验。用 QCM(Attana A200, 斯德哥尔摩, 瑞典) 评价天然人 IgG 和水凝胶释放的人 IgG 的生物活性。该组织由薄的压电石英圆盘组成, 在每一侧面都有电极。当连接到振荡电流时, 所述石英晶体以共振频率振动, 这对于结晶状物是敏感的, 当材料吸附到石英表面时, 可记录到频率发生变化。为了比较天然人 IgG 和水凝胶释放的人 IgG 的结合性质, 用胺耦合试剂盒将 PC-BSA 抗原共价固定到通路 A 和 B 上。初步实验显示在 0.05% 吐温清洁剂中非特异性结合最小, 因此, 所有的 IgG- 抗原结合研究都在此吐温浓度下进行。.

[0072] 通过注射 84 秒 35 μ E 的 20 微克 / 毫升人 IgG, 使其以 20 微升 / 分钟的速率流过固定在 QCM 表面上的 PC-BSA, 并测量由于 IgG 与 PC-BSA 的结合作用导致的表面重量增加, 从而获得室温条件下的动力学数据。参照阴性参照物矫正动力学数据, 所述阴性参照物通过注入以下观察到: (i) 将人多克隆 IgG 注入固定有 BSA 的表面上; (ii) 将单克隆抗 His 标记抗体注入固定有 PC-BSA 的表面上, 和 (iii) 将抗视紫红质抗体注入固定有 PC-BSA 的表面上。使用 Attache 评价软件分析数据, 利用简单的 1 : 1 结合模型模拟抗体和抗原之间的结合反应。通过分析相关性、 k_a 和解离, 计算 k_a 、速率常数以及结合亲和常数, 天然 IgG 和释放的 IgG 对于抗原的结合亲和常数定义为 $K_D = k_d/k_a$ 。根据 χ^2 值和接近于零的残留误差评价拟合度。

[0073] 结果和讨论

[0074] 人 IgG 通过肽纳米纤维脚手架水凝胶释放。如图 3A 所示, 在第一个小时内可以观察到 IgG 的最初快速释放 (爆炸效应)。这或许是由于位于溶剂 / 水凝胶接触面上的 IgG 分子或者在接触面附近的 IgG 分子快速释放入上清液溶液中。上述工作显示通过蛋白质这种肽水凝胶的释放取决于蛋白质的大小^[10]。小蛋白质释放的更快, 而 IgG 作为一种大蛋白质 ($\sim 150\text{kD}$), 释放的较慢。其中, 据显示, IgG 在三天后仍然没有完全释放。这里, 我们表明 IgG 通过肽脚手架水凝胶的释放不会再三个月后逐渐达到平稳值。在水凝胶系统中, 蛋白质的释放很少能达到 100%, 理由是蛋白质分子会被物理截留在高度卷曲的水凝胶纳米纤维结构域中, 使扩散剂不能自由运动。然而, 自组装肽水凝胶是可生物降解的, 因此, 在被引入到活的生物体中之后, 水凝胶将被分解成单个肽单体, 随后被分解成氨基酸。这一过程使脚手架水凝胶中装载的全部内含物都被释放到宿主组织中。

[0075] 肽脚手架水凝胶密度对人 IgG 扩散的作用。为了调查水凝胶密度对 IgG 释放曲线的作用, 改变自组装肽密度。增加的肽浓度导致纳米纤维高密度网络结构, 这回阻碍 IgG 的释放。图 3 显示了 IgG 穿过水凝胶的释放动力学, 所述水凝胶中含有 ac-(RADA)4-CONH2 肽水凝胶, 其中肽浓度为 0.5% 重量 / 体积 (99.5% 水)、1.0% 重量 / 体积 (99% 水) 和 1.5% 重量 / 体积 (98.5% 水), 和 ac-(KLDL)3-CONH2 肽水凝胶, 其中肽浓度为 0.3% 重量 / 体积 (99.7% 水) 和 0.6% 重量 / 体积 (99.4% 水)。没有对高密度 ac-(KLDL)3-CONH2 肽进行实验, 这是由于这种水凝胶的特点在于增加的刚性, 这种刚性使样品难以处理。这些结果显示可以 IgG 通过改变肽纳米纤维密度来控制 IgG 经过水凝胶的释放。

[0076] 构建用于药物释放的复合多层肽水凝胶结构。所述双层水凝胶系统由两个部分组成。图 A 和 2B-D 显示了两组分肽水凝胶的结构, 其中, 两组分中的每一个都装载有染料, 从而便于观察。所述水凝胶球的核心部分由自组装 ac-(RADA)4-CONH2 肽组成, 并且包含 CY3 染料 (红色), 而包覆密封的第二球由 ac-(KLDL)3-CONH2 肽组成, 并且包含 Alexa-488 (绿

色) 荧光团。亮场和荧光显微术显示每个水凝胶的轮廓(图 2B-D)。虽然染料的扩散最终导致 CY3 和 Alexa-488 分别扩散到 ac-(KLDL) 3-CONH2 和 ac-(RADA) 4-CONH2 水凝胶中, 并且可以预计在整个二组分肽水凝胶系统中均匀分布, 这些染料的初始分布清楚地定义了每个水凝胶球的形状和尺寸。

[0077] 在抗体释放实验中, 通过 1.0% 的 ac-(RADA) 4-CONH2 肽凝胶化作用形成核心, 向所述核心中装载抗体, 而第二层(壳)由 ac-(KLDL) 3-CONH2 水凝胶组成, 其中不包含抗体并且其包覆密封所述核心。多层水凝胶的形成产生一种系统, 在此系统中, 最初的蛋白质脉冲释放显著的小于在单组份水凝胶中所观察到的最初释放(图 3A 和 4A)。因此, IgG 通过多层水凝胶的扩散产生表面上接近-零-顺序的扩散曲线(图 4A&4B)。

[0078] 多层自组装肽水凝胶技术可以容易的实验室转化为临床应用, 通过使用例如具有同轴针的双组分注射器同时注射两个肽溶液。随着肽溶液与生物流体之间的相互作用, 发生凝胶化, 活性化合物的释放可以持续延长的时间。

[0079] IgG 通过水凝胶脚手架是扩散率。20°C 下, 无限稀释的 IgG 在水中的扩散系数可以通过下式计算 $0.4 \times 10^{-10} \text{m}^2 \text{s}^{-1}$ 使用史托-爱因斯坦方程 $D_{S_E} = k_B T / 6 \pi \eta \gamma_h$, 其中, k_B 是玻耳兹曼常数、 T 是介质的绝对温度、 η 是溶剂的动态粘度(认为是 1.002cP), 并且 γ_h 是 IgG 的流体动力学半径^[20]。然而应当指出, 当使用微摩尔的 IgG 浓度时, 如目前的工作, 单分子分析显示史托-爱因斯坦方程将 IgG 在溶液中的扩散率过高估计了 10-20%^[10]。进一步研究针对无限稀释分子的史托-爱因斯坦方程, 随后进行布朗运动, 因此微摩尔级浓度的分子可以通过缓慢的分子运动影响扩散。

[0080] 为了计算 IgG 在通过肽水凝胶释放期间的扩散系数, 我们使用图 3 显示的释放曲线。利用常用的 Fickian 模型, 如式 I 的描述, 会产生水凝胶内部的表观 IgG 扩散系数, 这种扩散系数与使用史托爱因斯坦方程确定的 IgG 在溶液中的扩散系数明显不同(即, 在 50% 到 80% 之间)。通过假定小分子扩散、扩散剂的无限稀释、和所述分子通过水凝胶的扩散只取决于布朗运动, 可以研发出使用菲克定律计算的扩散系数模型。等式 1 常用于确定表观扩散系数, 即使不使用这些情况时。这样做的理由是当没有其他可容易的确定扩散率的转化方法时, 它能够促进系统的讨论。在水凝胶系统中, 很少满足这些假设。将释放数据绘制成时间平方根的函数(图 3C), 绘图结果显示双相扩散机理。每个绘图最初的线性部分显示 IgG 通过所述肽水凝胶扩散控制的释放, 其可以根据菲克定律(等式 1) 计算扩散系数。长时间后偏离直线可能与非菲克、反常扩散有关。

[0081] 具有较小尺寸的水凝胶孔和/或扩散中的 IgG 分子和水凝胶的肽纳米纤维之间的特异性相互作用造成的扩散障碍都可以导致不符合菲克定律。

[0082] 释放的 IgG 的结构性质。蛋白质聚结作用以及导致蛋白质失活的蛋白质-肽相互作用会发生在 IgG 在所述肽溶液中滞留期间, 发生在自组装和纳米纤维形成期间或者发生在释放过程中。为了观察到从肽水凝胶中释放的 IgG 的构造状态, 在包裹密封后 2 个月进行远紫外圆二色性和荧光光谱分析分别检测二级和三级结构特性。

[0083] 天然 IgG 的 CD 广谱与文献中报道的 IgG 的光谱相同^[23]。如图 5A 所示, 水凝胶释放的 IgG 的 CD 光谱与新鲜制备的 IgG 溶液的光谱非常相似。在低于 200 纳米的一些谱图中观察到细微偏差, 这时二极管或者 HT(即, 总吸光度) 水平较高。在此区域广谱中观察到低信号-噪音比例的原因是相对低的释放的 IgG 浓度与可能存在的从脚手架上分离的纳

米纤维。但是, IgG 的 β - 片结构, 如在 218 纳米下观察到的, 在释放过程中不受影响。

[0084] 在 300 纳米条件下激发色氨酸并记录荧光发射光谱。该发射对蛋白质三维结构内的色氨酸微环境是敏感的, 因此, 荧光发射可以用于检测从水凝胶中释放的 IgG 的三级结构变化。在最大发射波长的红色位移显示蛋白质未折叠, 这是色氨酸暴露于极性溶液的结果。IgG 的荧光发射光谱与之前文献报告的相似 [24]。图 5B 显示在相同的 IgG 浓度下, 相对于二者的发射最大值和荧光强度, 释放的 IgG 发射光谱与天然 IgG 发射光谱相似, 这表明 IgG 的包覆密封和释放不会导致其三级结构改变。

[0085] 释放的人 IgG 的功能性实验。作为一种亚群, 人 IgG 已知能够结合磷酸胆碱 (PC), 这是这一种常见的存在于许多人感染的微生物 (包括肺炎球菌) 中的抗原 [25, 26, 27, 28], PC- 结合的 BSA 抗原被用于检测天然 IgG 和水凝胶释放的 IgG 的功能性。本实验中使用的 PC-BSA 样品中每个 BSA 包含 17 个磷酸胆碱 (PC) 分子。使用单分子 FCS 检测系统研究人 IgG 与 PC-BSA 之间的相互作用。通过使用一个和多个组分进行自相关函数拟合分析 GCS 数据。通过 χ 参数值和残差判断每组数据的拟合优度, 其在零周围均匀分布。在所有的情况下都选择最简单的模型。实验数据与具有较少独立组分的模型的尝试拟合会导致 χ^2 值增加。还要进行参照实验: (i) 缓冲液; (ii) 在抗原存在的情况下未标记的 IgG 分子; (iii) 在 PC-BSA 抗原存在的情况下, 热变性的标记的 IgG; 和 (iv) 在 BSA (无 PC 半抗原) 存在的情况下, 标记的 IgG, 并且未发现可测量的相互作用。

[0086] 数据分析显示, 一个 τ_D 足够描述游离 IgG 的自相关函数, 随着过量抗原的加入, 需要三个 τ_D 值进行 FCS 数据分析 (图 6)。随着 IgG 与 10 倍过量的 PC-BSA 抗原 (即, 50 微摩) 的相互作用, 根据其扩散时间可以识别出三种不同的种类, 游离地、未结合的 IgG 分子、和与一个或者两个抗原分子结合的 IgG 分子 (图 6)。数据分析显示, 无论是否存在抗原, 游离的 IgG 分子扩散通过检测体积的时间为 ca. 1060 微秒。比较 IgG 在通过肽水凝胶释放之前和之后的扩散时间说明, 随着与抗原的相互作用, 形成相同类型的 IgG-(PC-BSA) \times 复合物。这些复合物具有的扩散时间为大约 1135 微秒和 1230 微秒, 分别对应于结合 IgG 结合一个或者两个抗原分子。图 6 还显示在每个种类 (即, 游离分子和与一个或者两个抗原相互作用的分子) 中 IgG 分子的百分比在通过水凝胶释放之前和之后都是相似的。这些结果显示 IgG 与 PC-BSA 抗原的结合亲和力不会随着 IgG 从肽水凝胶中的释放而改变, 因此, IgG 的生物活性不受到水凝胶包覆密封和释放的影响。

[0087] 在多克隆人 IgG 样品中相对高浓度的抗-PC IgG 分子 (图 6) 促进进一步调查。据文献报道, 之前 Scott 等人通过使用放射免疫法已经观察到在人类血清中具有相当大比例的抗-PC-BSA 抗体, 及, 100-800 微克 / 毫升, 血液样品来自多个个体 [29]。根据这些值和 IgG 在人类血清中的浓度 (大约 8-16 毫克 / 毫升), 使用单分子分析观察到相对高百分比的抗-PC 抗体是合理的。在我们的实验中, PC-BSA 中每个 BSA 蛋白单位包含大约 17 个 PC 基团。这说明根据 FluoroPoint 观察到的结合不是非特异性结合, 而是抗-PC 免疫球蛋白与 PC-BSA 抗原的高亲和力结合的结果。需要进行进一步工作确定所观察到的是否可以在其他来源的人 IgG 样品中重现。

[0088] 为了检测人 IgG 通过肽脚手架水凝胶释放之前和之后的功能性, 使用 QCM 技术。IgG 功能性标准是单克隆 IgG 和 PC-BSA 抗原之间的结合动力学和亲和常数。随着与固定在 QCM 金表面上的 PC-BSA 的相互作用, 已经显示在肽水凝胶中 48 小时后、在一个月和两

个月后,释放的人 IgG 与天然 IgG 相比不发生功能性变化。随着如图 7 中所示的数据拟合,动力学分析显示天然 IgG 的相关性 $ka = 9.47 \pm 0.05 \times 10^4 M^{-1} s^{-1}$ 和解离速率常数 $kd = 1.65 \pm 0.02 \times 10^{-3} s^{-1}$ 与水凝胶释放的 IgG 所观察到的 $ka = 11.85 \pm 0.10 \times 10^4 M^{-1} s^{-1}$, $kd = 2.39 \pm 0.02 \times 10^{-3} s^{-1}$ 相似。而且,天然 IgG 和水凝胶释放的 IgG 的结合常数分别为 $KD = 17.4 \pm 0.2 nM$ 和 $KD = 20.2 \pm 0.2 nM$, 也是相似的。这些结果证实单分子分析并且表明在释放实验过程中,释放后抗体的功能性不受其与肽水凝胶相互作用的影响。

[0089] 结论

[0090] 持续药物释放的一个主要目的是有效的将治疗剂导向具体组织。在药物具有副作用的情况下,定位传递会对患者产生更少的毒性副作用。本发明的可注射自组装肽脚手架系统在生理条件下是凝胶态,是一种潜在的鲁棒系统能够用于免疫治疗剂的持续释放应用,从而在延长的时间段内在特定组织中局部释放活性抗体。在本研究中,已经显示人抗体只通过 ac-(RADA)4-CONH₂ 或者 ac-(KLDL)3-CONH₂ 肽水凝胶在超过 3 个月的时间内缓慢释放,并且释放动力学取决于形成水凝胶的自组装肽的氨基酸序列和水凝胶中肽纳米纤维的密度。一种有效的控制释放系统可以呈递生物活性蛋白质。检测水凝胶释放的抗体的二级结构和三级结构以及他们的生物活性,结果显示他们不会受到被水凝胶包覆密封和从水凝胶中释放的影响。这些结果提供了一种创造新型量身定制的并且可编程的肽水凝胶用于持续释放抗体和其他蛋白质的机会。肽水凝胶系统可以容易地被设计和合成从而控制 IgG 和其他治疗剂化合物的释放。肽序列的可编程性是独特的,并且提供了一种将纳米纤维性质控制在分子水平的方法,这反过来可以改变生物分子扩散和释放动力学。

[0091] 尽管本发明已经参照其优选的实施方案进行了具体显示和描述,但是本领域普通技术人员应该理解,在不脱离由所附权利要求限定的本发明的范围的情况下,其在形式和细节上可以进行多种变化。

[0092] 肽序列列表

- [0093] ac-(RADA)₂-CONH₂ (SEQ ID NO :1)
- [0094] ac-(RADA)₃-CONH₂ (SEQ ID NO :2)
- [0095] ac-(RADA)₄-CONH₂ (SEQ ID NO :3)
- [0096] ac-(RADA)₅-CONH₂ (SEQ ID NO :4)
- [0097] ac-(KLDL)₂-CONH₂ (SEQ ID NO :5)
- [0098] ac-(KLDL)₃-CONH₂ (SEQ ID NO :6)
- [0099] ac-(KLDL)₃-CONH₂ (SEQ ID NO :V)
- [0100] ac-(KLDL)₄-CONH₂ (SEQ ID NO :8)
- [0101] ac-(KLDL)₅-CONH₂ (SEQ ID NO :9)
- [0102] KLEALWLGFFGFFILGISYIR (SEQ ID NO :10)
- [0103] KLEALYIIMVLGFFGFFILGIMLSYIR (SEQ ID NO :11)

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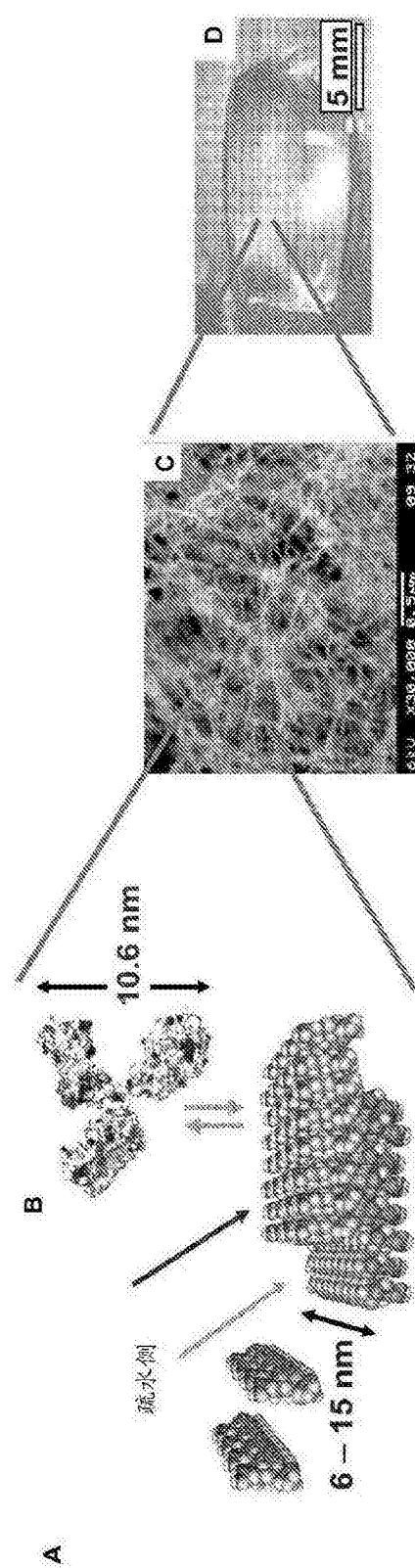


图 1

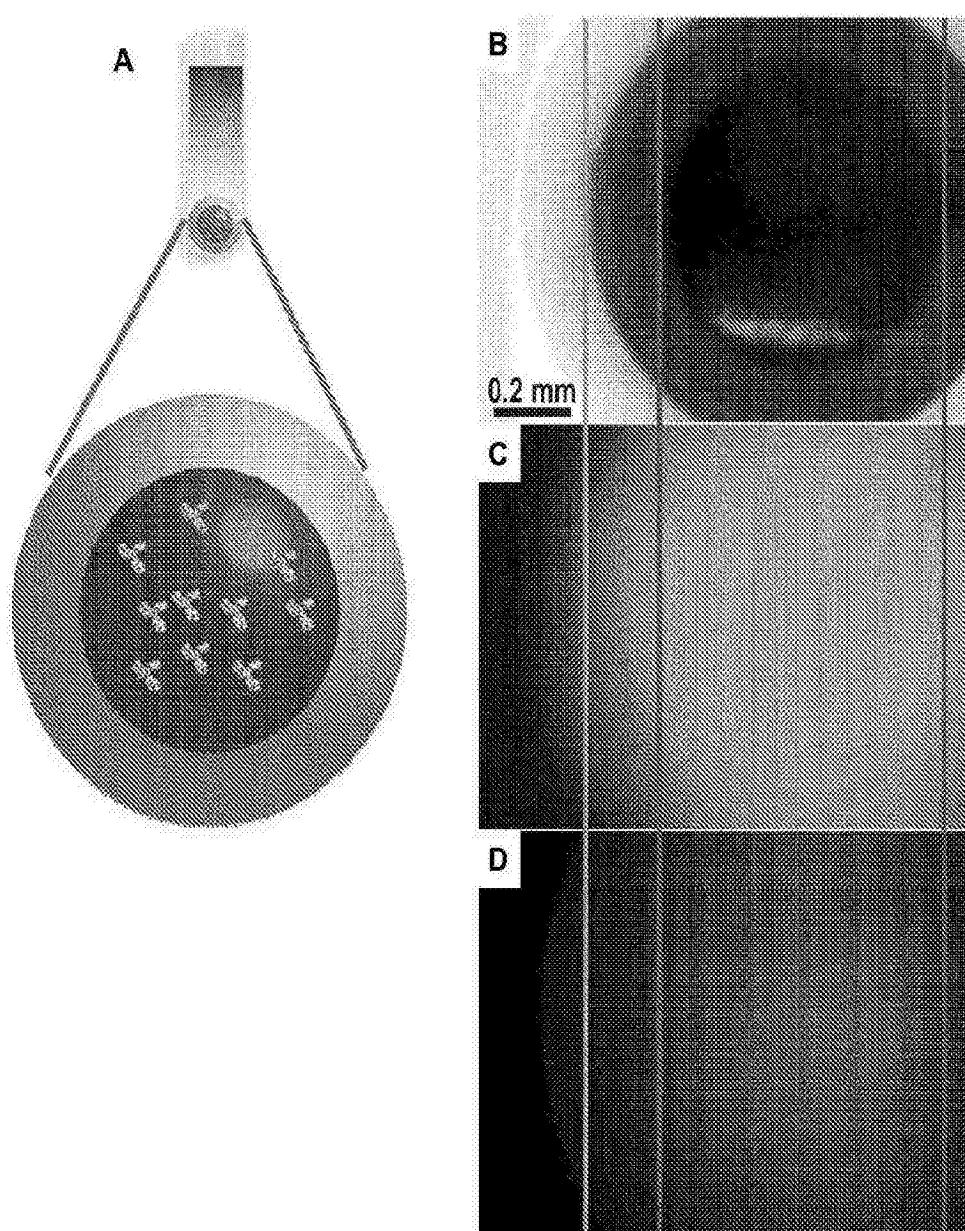


图 2

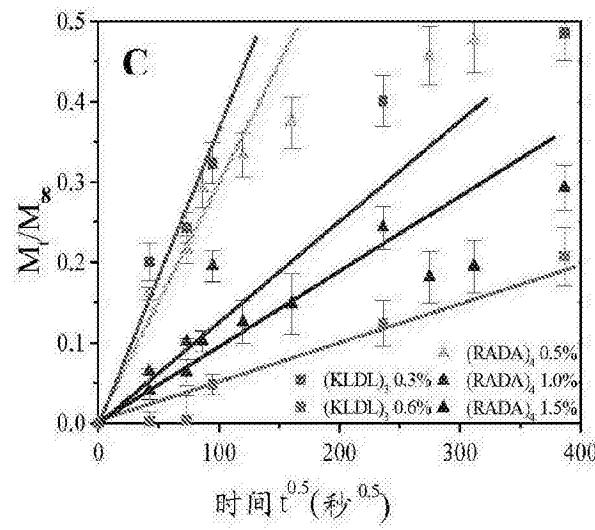
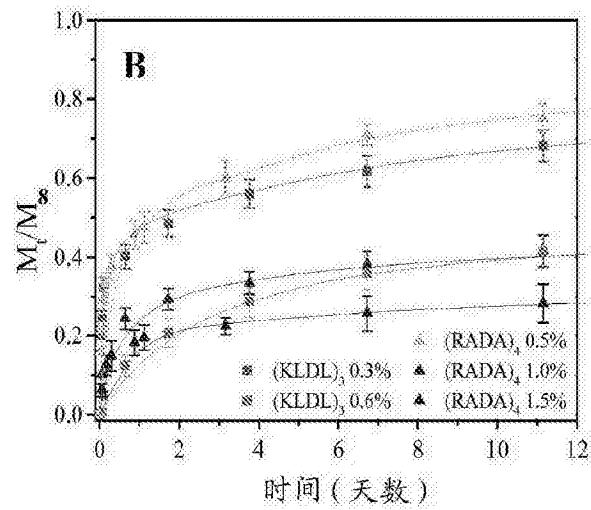
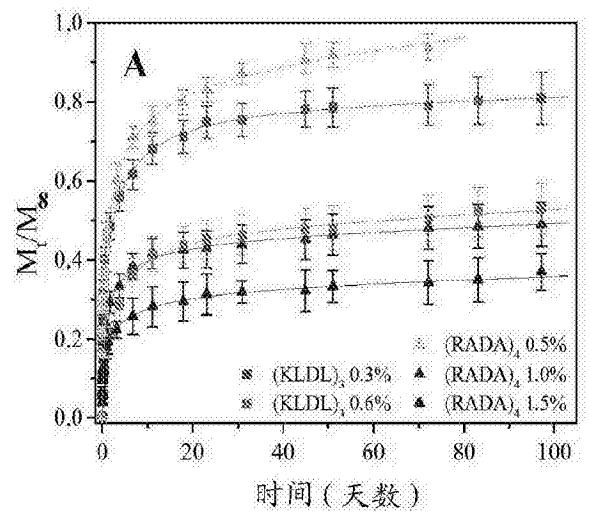


图 3

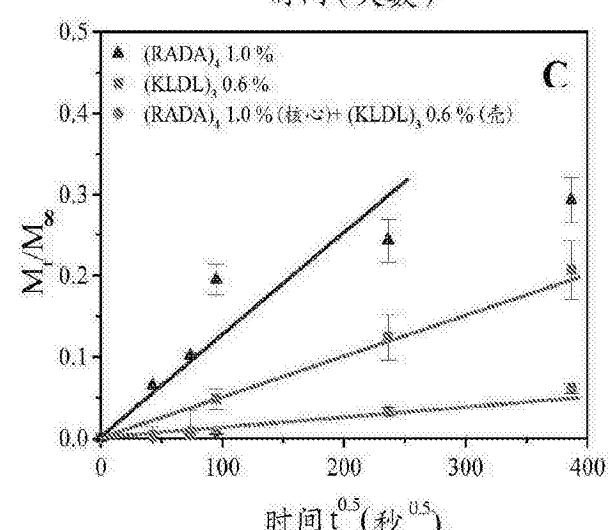
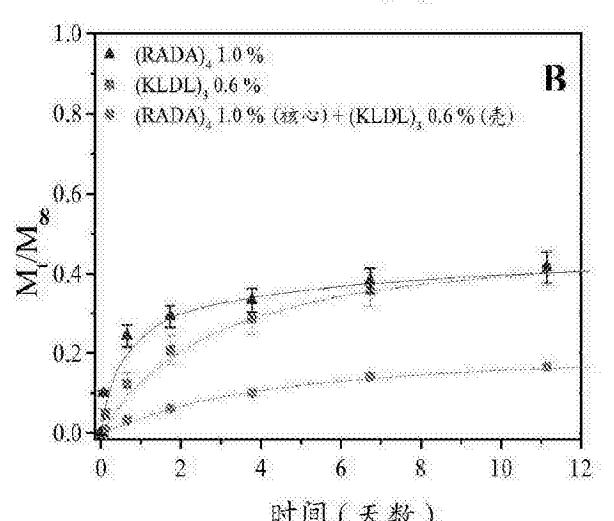
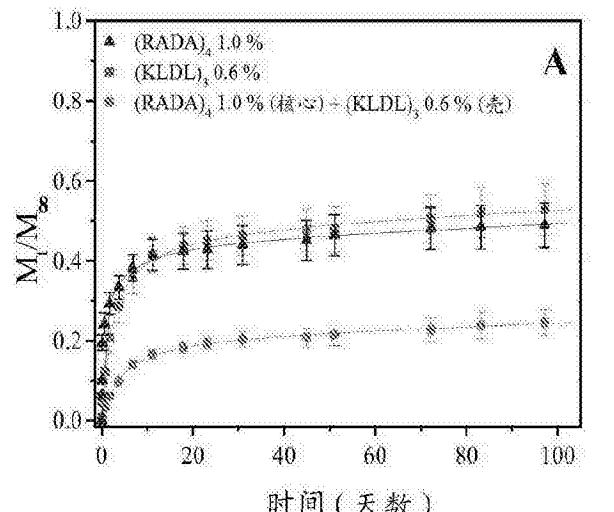


图 4

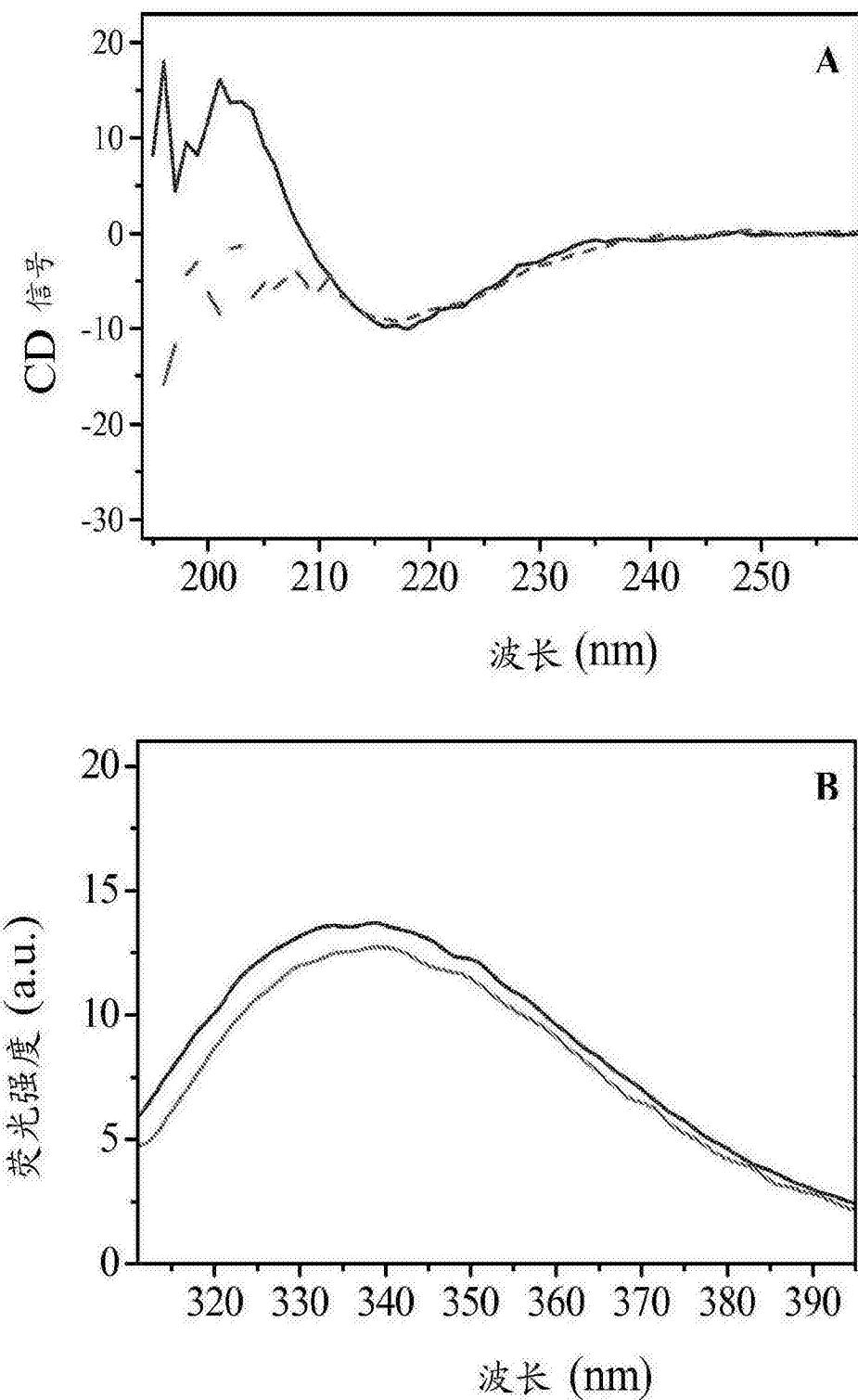


图 5

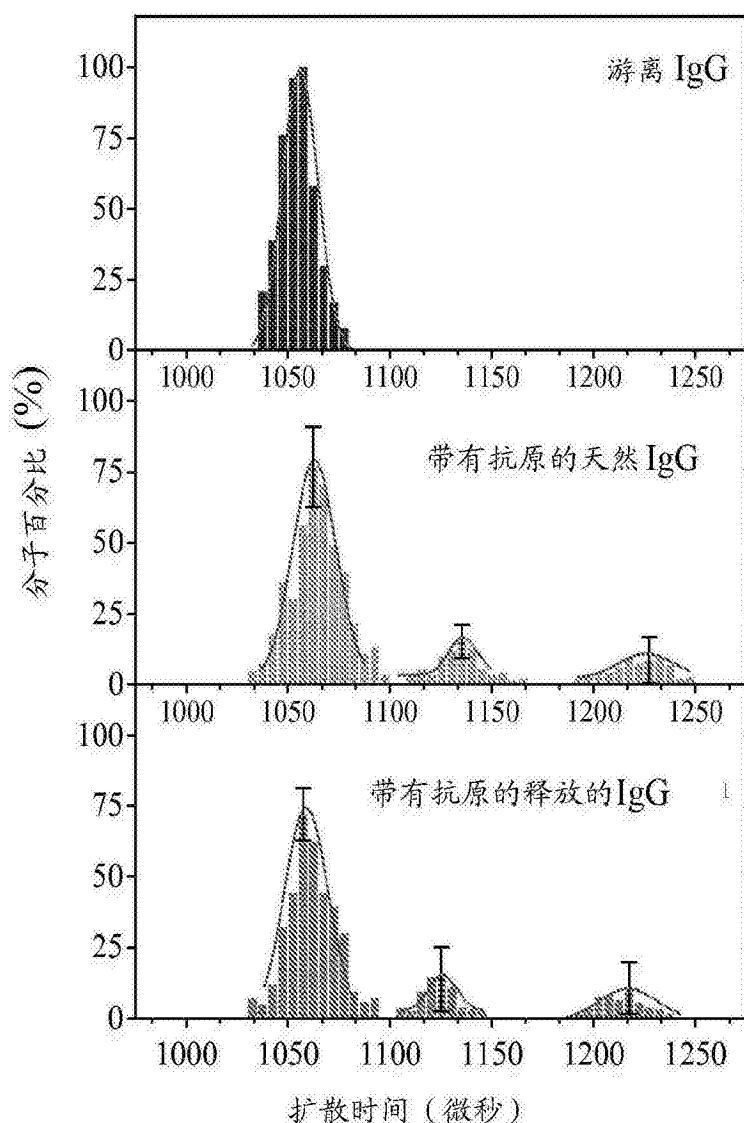


图 6

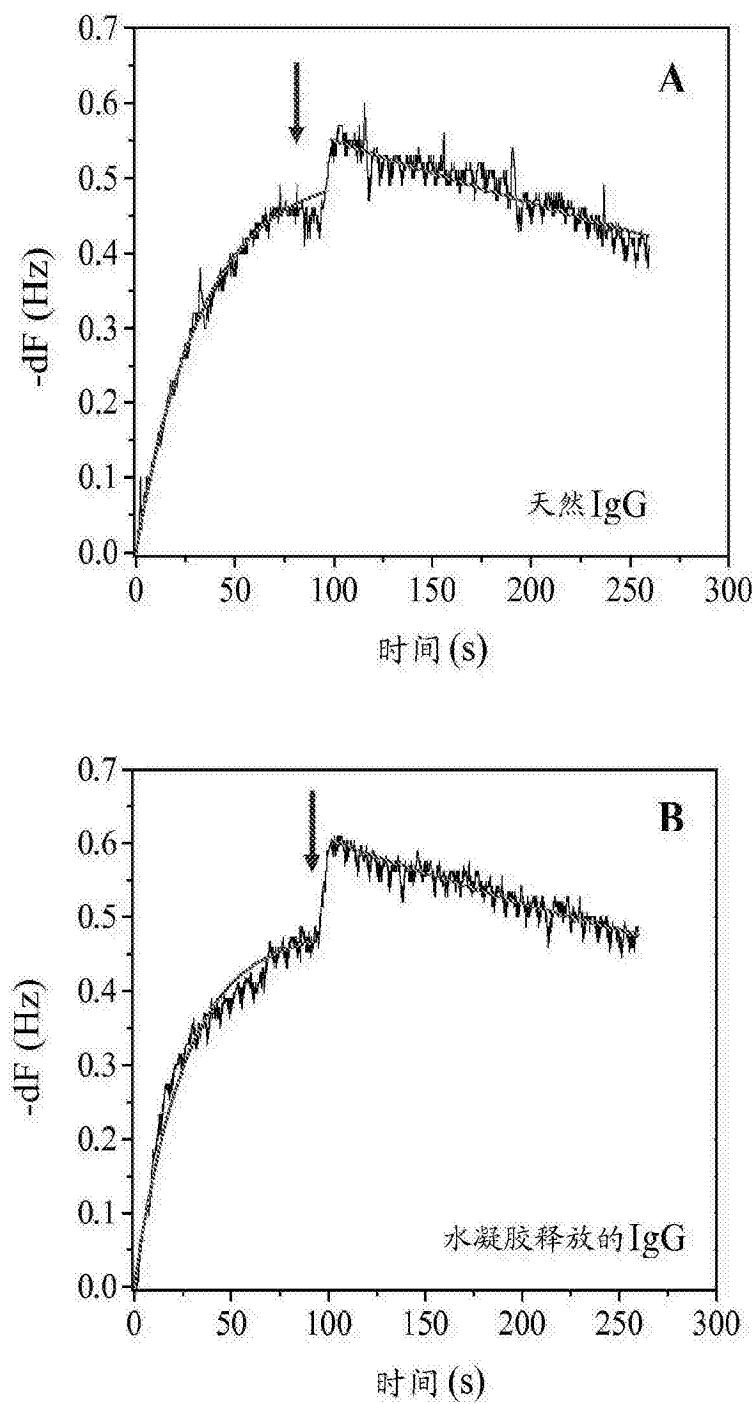


图 7

肽水凝胶	水凝胶 密度 (w/v)	扩散常数 ($10^{-10} \text{ m}^2/\text{s}$)	
		史托-爱因斯坦 估计	在凝胶中的表现 (等式 1)
	0.5%		0.183 ± 0.002
ac-(RADA) ₄ -CONH ₂	1.0%	0.4	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 (核心)	1.0% (核心)		
+	+	0.4	0.0010 ± 0.0001
ac-(KLDL) ₃ -CONH ₂ (壳)	0.6% (壳)		

图 8