(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
5 March 2009 (05.03.2009)

PCT

(10) International Publication Number
WO 2009/028764 A1

(51) International Patent Classification:
A61K 9/22 (2006.01) *A61K 47/48* (2006.01)
A61K 9/50 (2006.01)

(21) International Application Number:
PCT/KR2007/005656

(22) International Filing Date:
9 November 2007 (09.11.2007)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:
10-2007-0085750 24 August 2007 (24.08.2007) KR

(71) Applicant *(for all designated States except US)*: **INDUSTRY-UNIVERSITY COOPERATION FOUNDATION HANYANG UNIVERSITY** [KR/KR]; 17 Haeng-dang-dong, Seongdong-gu, Seoul 135-851 (KR).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KIM, Yong-Hee** [KR/KR]; 78-906, Hyundai Apt., Apgujeong-dong, Gang-nam-gu, Seoul 135-900 (KR). **JIN, Kwang-Mi** [KR/KR]; 98-2 bunji, Bongbang-dong, Chungju Si, Chungcheong-buk-do 380-080 (KR).

(74) Agents: **NAHM, Ho-Hyun** et al.; 5th Fl., D & M BUild-ing, 1003, Daechi-dong, Gangnam-GU, Seoul 135-851 (KR).

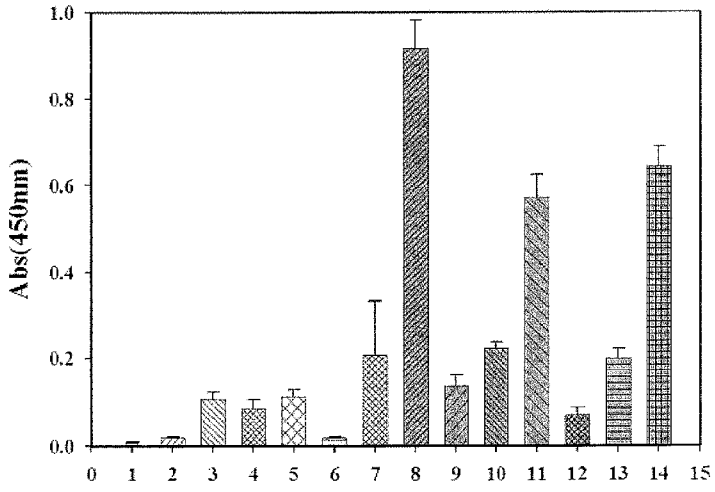
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

(54) **Title:** THERMO-REVERSIBLE COACERVATE COMBINATION GELS FOR PROTEIN DELIVERY

FIG. 6



(57) **Abstract:** The present invention relates to a combination gel of a complex coacervate and a thermo-reversible polysaccharide useful for protein drug delivery. The combination gel of the present invention for drug delivery comprises a complex coacervate of positive-chargedprotein andnegative-chargedpolysaccharide, and a negative thermo-reversible polysaccharide containing salting-out salt. The gel of the present invention is water-like sol form at room temperature which is easy to inject while after being injected into a body the formulation rapidly forms a gel and the gel provide slow and sustained release of the protein contained in the gel.

WO 2009/028764 A1

THERMO-REVERSIBLE COACERVATE COMBINATION GELS FOR PROTEIN DELIVERY

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a gel for drug delivery. More specifically, the present invention relates to a combination gel of a complex coacervate and a thermo-reversible polysaccharide useful for protein drug delivery.

Description of the Related Art

The most common and simple drug delivery system may be in the form of oral administration, injection or patch or the like. However, such conventional types of delivery systems just cannot serve the purpose of many types of new drugs as drug are becoming highly diversified recently.

Protein drugs are generally administered by oral route such as in the form of tablets or surgical route. Meanwhile, oral administration may face degradation of most of the protein drug to be delivered by intestinal enzymes, which fails to achieve the intended pharmacological effects. Surgery is not efficient in terms of the risks and costs.

Furthermore, a systemic delivery system such as oral administration has a critical disadvantage. Such a system delivers drug with the same concentration to undesirable sites that do not need the pharmacological effect, which can result in undesirable effects to the body.

Accordingly, there is increasingly needed a drug delivery system useful for targeting a disease site to treat the disease. It is also needed to provide a drug delivery system which may accurately act at a target site and at the same time give sustained drug release as long as possible to reduce the number of administration and risks from frequent injection.

In the past few years, *in situ*-forming systems have been reported for various biomedical applications including drug delivery and tissue engineering (See (R.L. Dunn et al., Biodegradable in-situ forming implants and methods of producing the same, US Patent 4 (1990) 938-763; B.O. Haglund et al., J. Control. Release 41 (1996) 229-235; and Y. An et al., J. Control. Release 64 (2000) 205-215).

Several mechanisms such as solvent exchange, pH change, UV-irradiation, ionic cross-linking and temperature transition has lead to in situ gel formation. Negative thermosensitive polymers with lower critical solution temperatures (LCSTs) or thermo-reversible hydrogels exhibiting reversible sol-gel transition behaviors upon heating or cooling are the most commonly

studied classes of stimuli-sensitive polymer systems for drug delivery. Although several synthetic polymers have been reported with thermo-reversible gelling behavior at body temperature and used for drug delivery, intrinsic problems in terms of biocompatibility and biodegradation remain.

High molecular weight natural celluloses are often insoluble in aqueous solutions due to intra- and intermolecular hydrogen bonds (Gels Handbook, Vol. 4; Osaka, Y., Kanji, K., Eds, Translated by Hatsuo Ishida; Academic Press: San Diego, CA (2001)).

When some of the hydroxyl groups were substituted for hydrophobic groups such as methyl or hydroxylpropyl, some of the hydrogen bonds were disrupted, resulting in water soluble, hydrophobically modified celluloses (See Guenet, J, Thermoreversible Gelation of Polymers and Biopolymers, Academic Press: London (1992); and Kabayashi, K. et al., Macromolecules 32 (1999) 7070).

Methylcellulose (MC) is a hydrophobically modified, non-ionic cellulose derivative that forms thermo-reversible physical gels in aqueous solutions. MC is a heterogeneous alternative block copolymer structure consisting of densely substituted hydrophobic regions and less- or unsubstituted hydrophilic regions (Kundu, K.K, Polymer. 42 (2001) 2015-2020). The hydrophobic region locally stabilizes water structures and upon heating, the water structures are disrupted leading to enhanced hydrophobic interaction and

consequent gellation. Commercial MC with a degree of substitution (DS) of 1.4-2.0 undergoes the sol- gel transition upon heating in the concentration range of 1.0-2.5% of MC in water. The phase-transition behavior has been widely applied to the food and pharmaceutical industries to be used as a binder or thickener.

Cellulase is an enzyme complex which breaks down cellulose to beta-glucose. Most animals, including humans, do not produce this enzyme and are therefore unable to independently use the plant energy. However, cellulases are widely abundant in fungal and microbial organisms and are used in human healthcare as a food supplement, which allows for hydrolyzation and break down of plant polysaccharides. Some fungi secrete enzymes that catalyze oxidation reactions of cellulose. The peroxidases can provide hydrogen peroxide for free radical attack on the C2-C3 positions of cellulose to form 'aldehyde' cellulose, which is very reactive and can further hydrolyze to lower molecular weight fragments. Bacteria also secrete endo- and exoenzymes for cellulose degradation to produce carbohydrate nutrients (Aubert, J.P., Beguin, P., Millet, J. Biochemistry and Genetics of Cellulose Degradation. Academic, New York (1988)).

In order for MC to be applied as an *in situ*-forming drug delivery system that gels at body temperature, the negative thermogelling behavior of MC needs to be modified with additives such as polymers,

nonelectrolytes, or salts. The gelling temperature of MC also decrease by increasing MC concentration, however the solution viscosity is so high that it is difficult to handle.

A simple way to modify the gelling temperature is to use salting-out salts that are known to have significant effects on the phase behavior of water-soluble polymers (Collins, K. D.; Washabaugh, M. W. Q Rev Biophys. 4 (1985) 323). Anions in salts are known to be more effective than cations for stabilizing the hydrophobicity of non-polar solutes and macromolecules. According to the Hofmeister(Collins, K. D.; Washabaugh, M. W. Q Rev Biophys. 4 (1985) 323) or lyotropic series(M. Khairy 등, Journal of Polymer Science (2003) 3547-3559), the salting-out strength of anions is as follows:

Hofmeister :

$SO_4^{2-} > HPO_4^{2-} > F^- > Cl^- > Br^- > NO3^- > I^- > ClO_4^- > SCN^-$

Lyotropic series :

$Al^{3+} > Ca^{2+} > Mg^{2+} > K^+ = NH_4^+ > Na^+ > Li^+$, $PO_4^{3-} > SO_4^{2-} > Cl^- > NO_3^-$

Coacervation is a process during which a homogeneous solution of charged macromolecules undergoes liquid-liquid phase

separation, producing a polymer rich dense phase. Two oppositely charged macromolecules (or a polyelectrolyte and an oppositely charged colloid) can undergo complex coacervates through electrostatic and additional interactions.

Coacervates have been widely applied to protein purification and drug delivery (Xia, J. 및 Dubin, P. L., Protein-polyelectrolyte complexes. In: Dubin, P. L., Bock, J., Davis, R., Schulz, D. N., 및 Thies, C., Eds. Macromolecular Complexes in Chemistry and Biology. Berlin: Springer-Verlag (1994) 247-271; Poznansky MJ, Juliano RL., Pharmacol Rev 36 (1984) 277-335; 및 Gombotz WR., Bioconjugate Chem 6 (1995) 332-51).

Complex coacervates can be spontaneously formed upon mixing oppositely charged polyelectrolytes in aqueous media. Protein-protein, protein-polysaccharide, and polysaccharide-polysaccharide combinations have been frequently studied for drug delivery and biomedical applications. For example, the preparation of microspheres by complex coacervation of gelatin A as a polycation or zwitterion and chondroitin 6-sulfate (CS) as a polyanion was reported to develop an intra-articular delivery system of therapeutic proteins (Kimberly EB, Arthritis&Rheumatism (1998) 2185-2195). As these polyions are major components of the extracellular matrix of cartilage and synovium, the microspheres were biocompatible and susceptible to matrix metalloprotease

(MMPs), which is induced by proinflammatory cytokines.

SUMMARY OF THE INVENTION

Accordingly, There is needed a drug or protein delivery system that can make up for the disadvantages of conventional drug delivery systems. Such system need to efficiently deliver a protein to a target site as well as provide sustained release of the protein for a long time at the target site to achieve the intended pharmacological effect.

The present inventors found that one may co-formulate a complex coacervates consisting of two oppositely charged biomacromolecules with thermo-reversible polymers, and such a formulation is water-like sol form at room temperature which is easy to inject while after being injected into a body the formulation rapidly forms a gel and the gel provide slow and sustained release of the protein contained in the gel. Therefore, the present invention provides a combination gel for drug delivery comprising a complex coacervate and a negative thermo-reversible polysaccharide and a preparation method thereof.

The present invention particularly provides a physical combinational gel, not chemically crosslinked, was hypothesized to be formed by adding two oppositely charged biomacromolecules

composed of natural or therapeutic proteins and polysaccharides, which results in complex coacervates. By further co-formulating the complex coacervates with thermo-reversible polymers, an injectable and intelligent system may be prepared. An optimized novel delivery system containing dual advantages of complex coacervation and temperature responsiveness was introduced and the potential for an efficient protein drug delivery system was demonstrated.

The present invention will be hereinafter described in detail.

In an embodiment the present invention provides a combination gel for drug delivery comprising a complex coacervate of positive-charged protein and negative-charged polysaccharide, and a negative thermo-reversible polysaccharide containing salting-out salt.

The kinds of drug to be effectively delivered by the present invention are not limited. Any drug, which needs to be delivered into the body, may be delivered using the gel of the present invention. Preferably, the drug is a protein or gene preparation for treating a disease.

The positive-charged protein is any protein that is commonly used for forming a complex coacervate. Examples of positive-charged proteins that may be preferably used in the present invention are gelatin, cow pancreas trypsinogen, egg lysozyme, basic fibroblast

growth factor, fibronectin, agrecan and casein. Most preferably the positive-charged protein is gelatin.

The negative-charged polysaccharide is any natural polysaccharide that is commonly used for forming a complex coacervate. Examples of negative-charged polysaccharides that may be preferably used in the present invention are chondroitin sulfate, heparin/heparan sulfate (HS), hyaluronan, dermatan sulfate and keratan sulfate. Most preferably the negative-charged polysaccharide is chondroitin-6-sulfate.

The ratio of the positive-charged protein to the negative-charged polysaccharide to form the most stable complex coacervate may be determined by using any method know to the one with ordinary skill in the art. Preferably, the content ratio may be determined by measuring the turbidity of the formed coacervate mixture.

Examples of negative thermo-reversible polysaccharides that may be preferably used in the present invention are methylcellulose (MC), hydroxyprophyl methylcellulose (HPMC), ethyl hydroxyethyl cellulose (EHEC), carrageenan, scleroglucan. Preferably the negative thermo-reversible polysaccharide is methylcellulose.

In the present invention a salting-out salt may be added into the negative thermo-reversible polysaccharide to lower the gelling temperature. Examples of salting-out salt that may be preferably used in the present invention are ammonium sulfate, sodium dodecyl

sulfate, glycerophospate, sodium carbonate, sodium perchlorate, sulfuric acid, sodium hydrogen carbonate. Salting-out salt to be used may be selected depending on the kind and properties of the polysaccharide. Most preferably, the salt is ammonium sulfate in the present invention.

In another embodiment the present invention provides a method of preparing a gel for drug delivery comprising the steps of mixing positive-charged proteins with negative-charged polysaccharides to form a complex coacervate; and mixing the complex coacervate with negative thermo-reversible polysaccharides containing salting-out salt.

The condition for forming a complex coacervate and the combination gel of a complex coacervate and a negative thermo-reversible polysaccharide may be well known in the art. The condition may vary depending on the choice of the constitutional elements of the gel to be mixed using different temperatures and buffers. One with ordinary skill in the art may form a stable coacervate by changing and selecting the conditions depending on particular constitutional elements to be used.

In another embodiment the present invention provides a method of preparing a combination gel for drug delivery comprising the steps of mixing positive-charged proteins with negative-charged polysaccharides to form a complex coacervate; mixing the complex

coacervate with negative thermo-reversible polysaccharides containing salting-out salt; and mixing a drug to be delivered.

By using the present drug delivery system consisting of a complex coacervate and a negative thermo-reversible polysaccharide, protein drugs may be effectively delivered into the body.

Furthermore, the thermo-reversible complex coacervate gel prepared according to the present invention may be easily produced using physical combination rather than chemical cross-linking reactions, which makes the gel biocompatible and biodegradable and make the preparation reaction simpler.

The drug delivery system of the present invention is easy to administer and is advantageous in terms of toxic solvent-free aquatic environment.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 and 2 graphically show the turbidimetric titration of gelatin A, B - CS mixture. Fig. 1 and 2 are turbidimetric titration curves of gelatins (Fig. 1 gelatin A, Fig. 2 gelatin B) and CS mixtures at various concentrations in PBS (pH 7.4) at 25°C (● : 6H, ○ : 12H, ▼ : 24H)

Fig. 3 illustrates the viscosity and gelation time measuremented by a rheometer at 25℃and 37℃. The viscosity and gelation time are represented by the storage modulus to signify elastic storage

of energy as the strain is recoverable in an elastic solid as an elastic component and the loss modulus to describe viscous dissipation(loss) of energy through permanent deformation in flow, respectively.

Fig. 4 shows MC solution gelation temperature as an effect of AS concentration. Gelation temperatures of 2% MC solutions are graphically illustrated as a function of AS concentration.

Fig. 5 and 6 show the time course of turbidity measured after the complex coacervate is formed between high molecular weight gelatin(HMw GA) and CS. Fig. 5 shows turbidimetric titration curves of high molecular weight GA and CS at various concentrations in PBS, pH 7.4 (●:10min, ○:30min, ▼:1h, △:3h, ■:6h, □:12h, ◆:1d, ◇:2d). Fig 6 is a bar graph of the measurement results of the turbidity of complex coacervate and thermosensitive gel. (1 : HMw GA, 2: HMw GA/AS, 3 : MC/AS, 4 : HMw GA/MC, 5: HMw GA/MC/AS, 6 : CS/HMw GA(1), 7 : CS/HMw GA(1)/AS, 8 : CS/HMw GA(1)/MC/AS, 9 : CS/HMw GA(0.1), 10 : CS/HMw GA(0.1)/AS, 11 : CS/HMw GA(0.1)/MC/AS, 12 : CS/HMw GA(0.01), 13 : CS/HMw GA(0.01)/AS, 14 : CS/HMw GA(0.01)/MC/AS)

Fig. 7 graphically shows the Viscosity and gelation time as a function of temperature. The graph represents the viscosity of the gel formulations comprising CS/HMw GA/MC/AS(0.4% HMw GA, CS/HMw GA(1), 2% MC, 9% AS) with and without coacervates at 25℃ and 37℃.

Fig. 8 shows the results of Gelatin release test. (●: HMw GA/MC/AS, ○: CS/HMw GA(2)/MC/AS, ▼: CS/HMw GA(1)/MC/AS) In the graph, release rate of GA from MC combination gels containing a coacervate at the optimum ratio of 0.1 between GA and CS was compared to the MC gel.

Fig. 9 illustrates the result of release test of negative thermo-sensitive gels containing HMw gelatin A-CS coacervate. 4% gelatin was used. (△: HMw GA/MC/AS, ● CS/HMw GA(1)/MC/AS, ▼ CS/HMw GA(0.5)/MC/AS, ○ CS/HMw GA(0.1)/MC/AS, ■ CS/HMw GA(0.05)/MC/AS, □ CS/HMw GA(0.01)/MC/AS, ◆MC/AS) Release profiles of a model protein, FITC-BSA, from gels indicating sustained release patterns over 25 days.

Fig. 10 shows photos of spherical depot that immediately formed after the injection of thermoreversible complex coacervate gel ((a) MC/AS, (b) MC/AS/GA, (c) MC/AS/CS/HMw GA(=1)). When various MC gel formulations were subcutaneously injected into the back of rat, immediate spherical depot formations were observed.

DETAILED DESCRIPTION OF THE INVENTION

Injectable, biodegradable, and thermo-reversible physical gels of the present invention were prepared using complex coacervates between two oppositely charged biomacromolecules and subsequently

co-formulating with a negative thermosensitive polysaccharide containing a salting-out salt.

Immediate spherical depots were formed after subcutaneous injection of the gels of the present invention into a rat, and the shape and rigidity of the depot were well maintained for one week.

The combination of complex coacervation and thermo-reversible gel demonstrated synergistic effects on the release rate of a protein and *in situ* gel depot formation. Gels showed sustained release patterns of a model protein over 25 days with minimal initial bursts. In order for sustained protein release, a stable complex coacervates with oppositely charged polyelectrolytes depending on their isoelectric points (pI) was suggested and concomitantly formulated with thermosensitive polysaccharide (MC in an embodiment of the present invention) gels.

Optimized novel *in situ* gel depot systems containing the dual advantages of complex coacervation and temperature responsiveness were developed in the present invention. These systems demonstrate a potential for efficient protein drug delivery, in terms of ease of administration, an aqueous environment without toxic organic solvents, and a simple fabrication method.

1. Complex coacervate formation between GA and CS

As a simple method to evaluate the polyionic complexation (complex coacervation) between gelatins and CS at pH 7.4, the turbidity of mixed solutions was measured by the absorbance at 450 nm to find optimum ratios of gelatins and CS for the stable complex coacervate formation.

Figures 1 and 2 show turbidimetric titration curves of gelatins and CS mixtures at various concentrations in PBS (pH 7.4) at 25°C. When cationic GA was used for the titration (Fig. 1), the mixed solution exhibited a maximum turbidity at the CS/GA weight ratio of 0.1, which increased as the complexation time increased. However, as expected, anionic GB did not form coacervates with CS at pH 7.4 (Fig. 2).

Phase separation has been reported by Bungenberg de Jong in which two oppositely charged molecules interact and the solvent forms a poor biopolymer phase (Kimberly EB, *supra*). The main interaction of biopolymers is electrostatic dependence on PH and ion strength values, while the significant factor affecting complex coacervate is charge.

Figure 3 shows the viscosity and gelation time of a GA and CS coacervate measured by a rheometer. As the mixture of GA and CS formed coacervates, the viscosity rapidly increased at 25℃ However, when the mixture was heated to 37℃ the viscosity noticeably

decreased and the coacervate gel transformed to a sol. As gelatin is a positive thermo-sensitive biopolymer showing decreasing viscosity with increasing temperature, the protein chain mobility of gelatin increases at 37℃ which results in the destruction of the coacervate gel between the GA and CS. To this end, this method was used for the remaining experiments.

2.1. Preparation of negative thermo-reversible MC gels

In order for complex coacervates as described above to be applied in a drug delivery system, especially for an *in situ* forming gel, the complexes need to be stable and form instant viscous gels at body temperature. The MC gels were tested as injectable formulations with incorporated complex coacervates. At room temperature, the aqueous MC solution was a transparent sol. Upon heating to approximately around 50 °C, the solution was an opaque gel. To increase practicality, the MC gelling temperature could be decreased further by controlling concentrations or using additives such as polymers, nonelectrolytes, or salts. The method of decreasing gelling temperatures below the body temperature by increasing the MC concentration limits the viscosity such that it was too high to be handled.

To this end, the addition of a salting-out salt, such as AS, was a simple and efficient method to decrease the gelling temperature of the aqueous MC solution.

Figure 4 shows the gelation temperature of 2 wt% MC solutions as a function of AS concentration. As the concentration of AS increased, the gelation temperature significantly decreased such that the gelation temperatures with 5% and 9% MC were 37 ℃and 29 ℃ respectively.

2.2. Optimization of thermo-reversible gels containing complex coacervates

In order to prepare thermo-reversible gels containing GA-CS complex coacervates, mixtures of GA and CS with various ratios were added to the 2 wt% MC solution containing 9% AS, which transformed from a sol to a gel near body temperature.

It was difficult to form stable gels at body temperature when GA was mixed with MC as the commercially available GA contained a heterogeneous molecular weight distribution. To this end, high molecular weight GA was prepared by ethanol desolvation and used to prepare MC gels containing complex coacervates.

Figure 5 shows turbidimetric titration curves of high molecular weight GA (HMw GA) and CS at various concentrations in PBS, pH

7.4. When high molecular weight GA was used for the titration, the mixed solution exhibited a maximum turbidity, which increased with complexation time.

As shown in Figure 6, the turbidity significantly increased as the stable complex coacervates of high molecular weight GA/CS were formed at an optimum weight ratio of 0.1. Interestingly, when MC was mixed with coacervates, the turbidity was higher than the coacervates, demonstrating the synergistic effect of MC on complex coacervate formation.

The viscosities of various gel formulations with and without coacervates were compared at 25℃and 37℃ Mixture solutions of MC and AS with and without CS/GA coacervates at optimum ratios were sol at 25℃ and a stable gel at 37℃. However, the viscosities of gels with coacervates were lower than plain gels, possibly due to increased flexibility by the gelatin.

3. In vitro release study

3.1 FITC-BSA release test

As shown in Figure 9, release profiles of the model protein, FITC-BSA, from the gels indicated a sustained release pattern over 25 days with minimal initial bursts. Although there was no significant difference in release rates among gels containing

various ratios of CS and GA, release rates from the combination gels containing coacervates were slightly faster than the plain MC and AS gel, which was predicted by the viscosity measurements. However, it is worthwhile to mention that the release profile from the combination gels containing coacervates indicated a long-term, sustained pattern and the gel became more flexible which may reduce pain and inflammation after subcutaneous injection. Approximately 30 days after the release experiment, the gels transformed into clear solutions.

3.2 Gelatin A release test

As the FITC-BSA release and viscosity results indicated that the incorporation of GA and CS coacervates reduced the viscosity of the MC gel and increased the release of FITC-BSA relative to the plain MC gel, it was hypothesized that the release rate of a model protein may be retarded when a stable complex coacervates was formed with oppositely charged polyelectrolytes and concomitantly added to the MC gels.

In order to test this hypothesis, the release rate of GA as a model protein from the MC combination gels containing GA/CS coacervates was measured. As shown in Figure 8, the release rate of GA from a MC combination gel containing a coacervate at a CS/GA

ratio of 0.1 was slower than the plain MC gel and much slower than the MC combination gel containing a coacervate at the non-optimum ratio. The reduced release rates indicate synergistic effects of complex coacervates and thermo-reversible gels on the protein controlled release.

4. In vivo gel depot formation

As shown in Fig. 10, when various MC gel formulations [(a) MC+AS, (b) MC+AS+GA, and (c) MC+AS+CS/GA(=0.1)] were subcutaneously injected into the back of rat using a 26GX 1/2 gauge syringe, immediate spherical depot formations were observed. The shape and rigidity of the depot was well maintained with a MC combination gel containing a coacervate (MC+AS+CS/GA(=0.1)).

The present invention will be described hereinafter in greater detail by way of non-limiting examples.

Examples

Materials & reagents

Methylcellulose (MC, M_n = 86,000, viscosity = 4,000 cps), gelatin type A (GA, PI = 8.6, from porcine skin derived from acid-cured tissue, Bloom No. 300), gelatin type B (GB, PI = 4.7, from bovine skin derived from lime-cured tissue, Bloom No. 225), chondroitin

6-sulfate (CS, derived from shark cartilage), fluorescein isothiocyanate conjugated bovine serum albumin (FITC-BSA), and ammonium sulfate (AS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were analytical grade.

Example 1: Complex coacervate formation and turbidity measurements

1.1. Preparation of high molecular weight gelatin solution

A high molecular weight gelatin solution was prepared by a modified ethanol desolvation method. GA was dissolved in PBS at 50℃ and the same volume of ethanol was added to desolvate and precipitate high molecular weight gelatin. The supernatant was discarded and the precipitate was redissolved in PBS at 50℃

1.2. Complex coacervate formation and turbidity measurements

GA or 4 wt% high molecular weight GA was dissolved in PBS, pH 7.4 at 50℃ for one h. CS in PBS, pH 7.4 was mixed with gelatin solutions at various weight ratios (CS/GA = 0.002, 0.01, 0.02, 0.1, 0.2 and 1.0). The increase in turbidity of the mixture solutions was measured by absorbance at 450 nm (DU 730, Life science UV/Vis spectrophotometer, Beckman Coulter, Fullerton, CA, USA).

Example 2: Preparation of thermoreversible complex coacervate

combination gels

2.1. Preparation of methylcellulose aqueous solution

Pure 0.3-2.5 wt% MC solutions were prepared by dispersing and stirring MC powders in approximately half of the required volume of phosphate buffered saline (PBS) preheated at 90℃ until the powder was thoroughly wet. The remaining volume of cold PBS was added and the mixture was gently stirred for 30 min in an ice bath to produce a colorless and transparent solution. The solution was shaken well and refrigerated.

2.2. Preparation of thermoreversible complex coacervate combination gels

The gelation temperature of 0.3-2.5 wt% MC aqueous solutions were measured by tube inverting method over a temperature range of 25-60℃ at a 0.5°C increase per 5 min interval. A salting-out salt agent, AS, was added to the MC solutions and the effect gelation temperature was determined by the same method.

In order to make combination gels, complex coacervates composed of cationic GA and anionic CS were added to MC solutions containing AS. Mixed solutions were vortexed for 5-10 min at room temperature and incubated in a water bath at 37°C.

Example 3: Gel formation and viscosity measurements

Gel formation and viscosity at various temperatures were measured by an oscillatory rheometer (Bohlin, Malvern, UK) using a cone and plate geometry in the temperature range of 0-60℃ Samples (500 ㎕) placed between two 20 mm parallel plates separated by a 500 ㎛ gap. The gap temperature was set at 25or 37℃ The phases of the combination gel were classified into three stages: clear solution, translucent gel, and phase separation.

Example 4: *In vitro* release study

The release profiles of coacervated GA and FITC-BSA from combination gels were measured at 37°C. The combination gels were prepared using complex coacervates with 4 wt% high molecular weight GA and 0.4 wt% CS, and co-formulating with 2 wt% MC containing 9 wt% AS. FITC-BSA was added to the aqueous gel solution. The sample vials were placed in a water bath at 37°C to form gels in which pre-warmed PBS, pH 7.4, covered and shaken at 150 rpm. At pre-determined time intervals, release media was withdrawn and replaced with fresh buffer. The protein concentration was measured by a UV spectrophotometer.

Example 5: *In vivo* experiments

Sprague-Dawley rats (200-250g, SLC, Tokyo, Japan) were anesthetized with the intramuscular administration of ketamine hydrochloride (90mg/kg) and xylazine hydrochloride (5mh/kg). All care and handling of the animals was performed according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH publication 85-23, revised 1985).

Samples (0.5 ml) with various formulations [(a) MC+AS, (b) MC+AS+GA, and (c) MC+AS+CS/GA (0.1)] were subcutaneously injected into the back of the rat using a 26G X 1/2 gauge, sterile hypodermic syringe. The presence of gel was observed 30 min after injection and retrieved.

What is claimed is:

1. A combination gel for drug delivery comprising (a) a complex coacervate of positive-charged protein and negative-charged polysaccharide and (b) a negative thermo-reversible polysaccharide containing salting-out salt.

2. The gel according to claim 1, wherein the drug is protein preparation.

3. The gel according to claim 1 or 2, wherein the positive-charged protein is gelatin.

4. A gel according to claim 1 or 2, wherein the negative-charged polysaccharide is chondroitin-6-sulfate.

5. A gel according to claim 1 or 2, wherein the negative thermo-reversible polysaccharide is cellulose derivative.

6. A gel according to claim 5, wherein the cellulose derivative is methylcellulose.

7. A gel according to claim 5, wherein the salting-out salt is

ammonium sulfate.

8. A method of preparing a gel for drug delivery comprising the steps of:

(a) mixing positive-charged proteins with negative-charged polysaccharides to form a complex coacervate; and

(b) mixing the complex coacervate in step (a) with negative thermo-reversible polysaccharides containing salting-out salt.

9. The method according to claim 8, wherein a drug to be delivered is mixed to the resulting mixture of step (b).

10. The method according to claim 8 or 9, wherein the drug is a protein.

11. The method according to claim 8 or 9, wherein the positive-charged protein is gelatin.

12. The method according to claim 8 or 9, wherein the negative-charged polysaccharide is chondroitin-6-sulfate.

13. The method according to claim 8 or 9, wherein the negative

thermo-reversible polysaccharide is cellulose derivative.

14. The method according to claim 13, wherein the cellulose derivative is methylcellulose.

15. The method according to claim 13, wherein the salting-out salt is ammonium sulfate.

FIG. 1

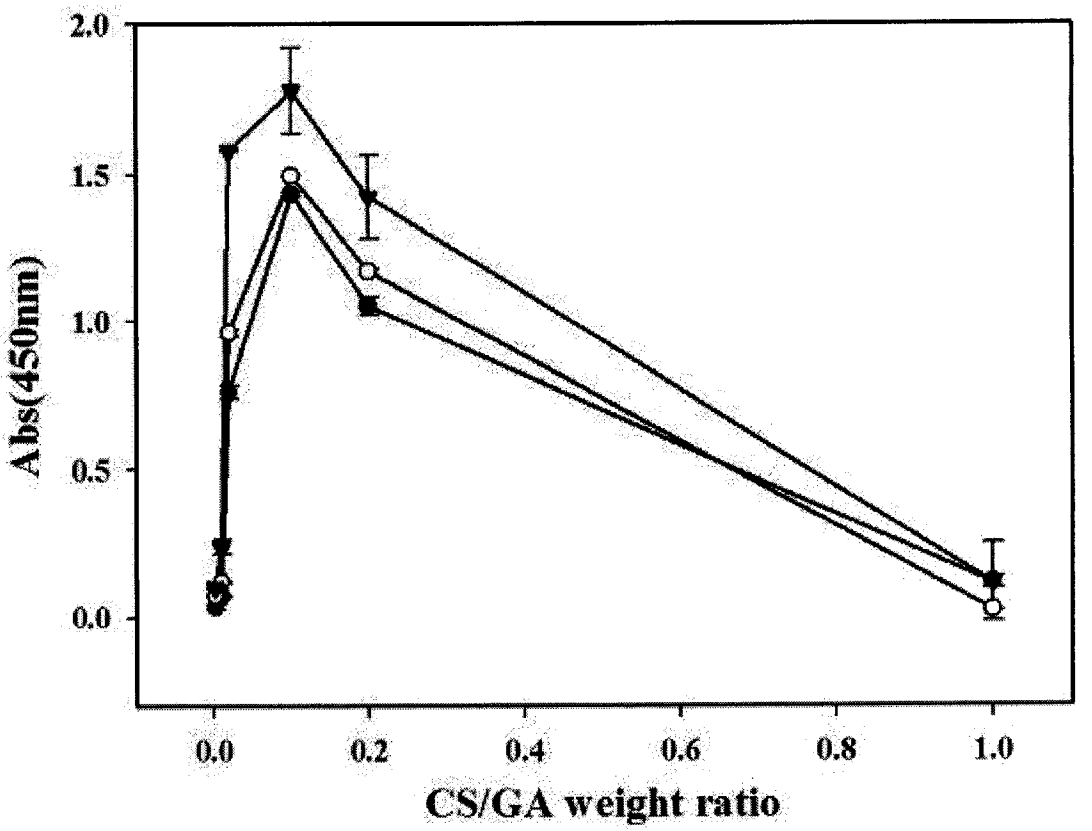


FIG. 2

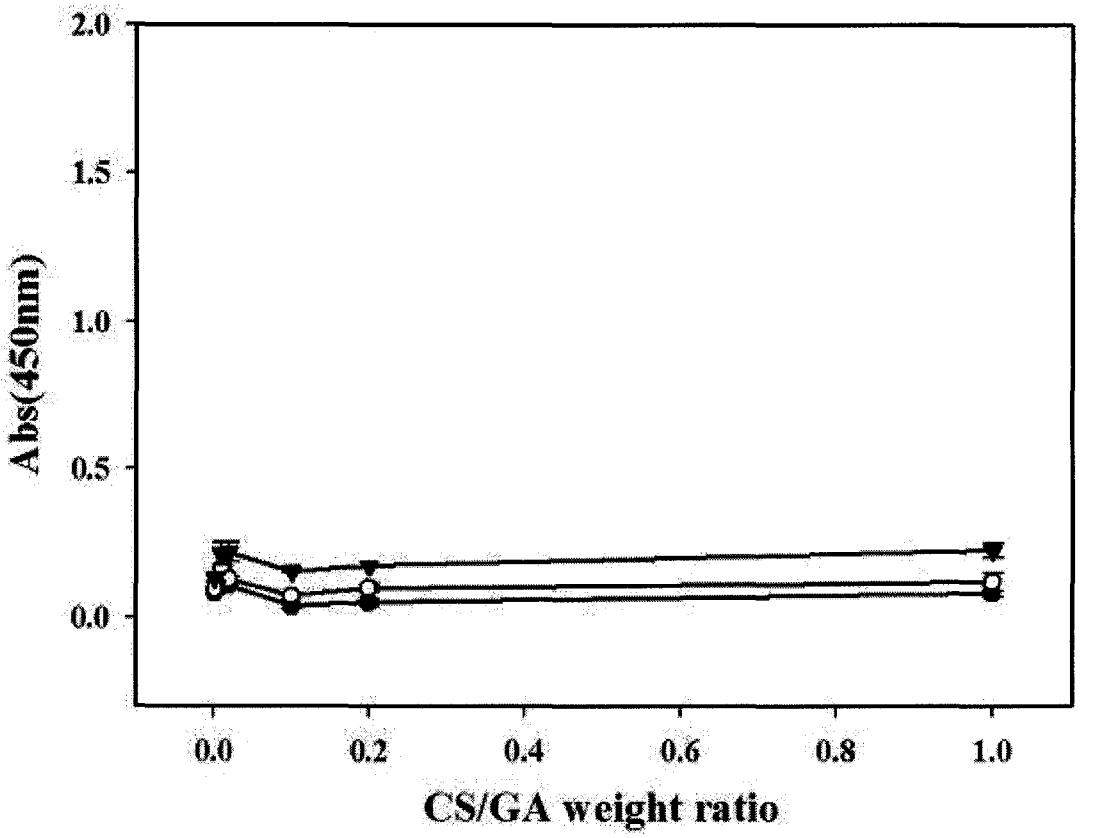


FIG. 3

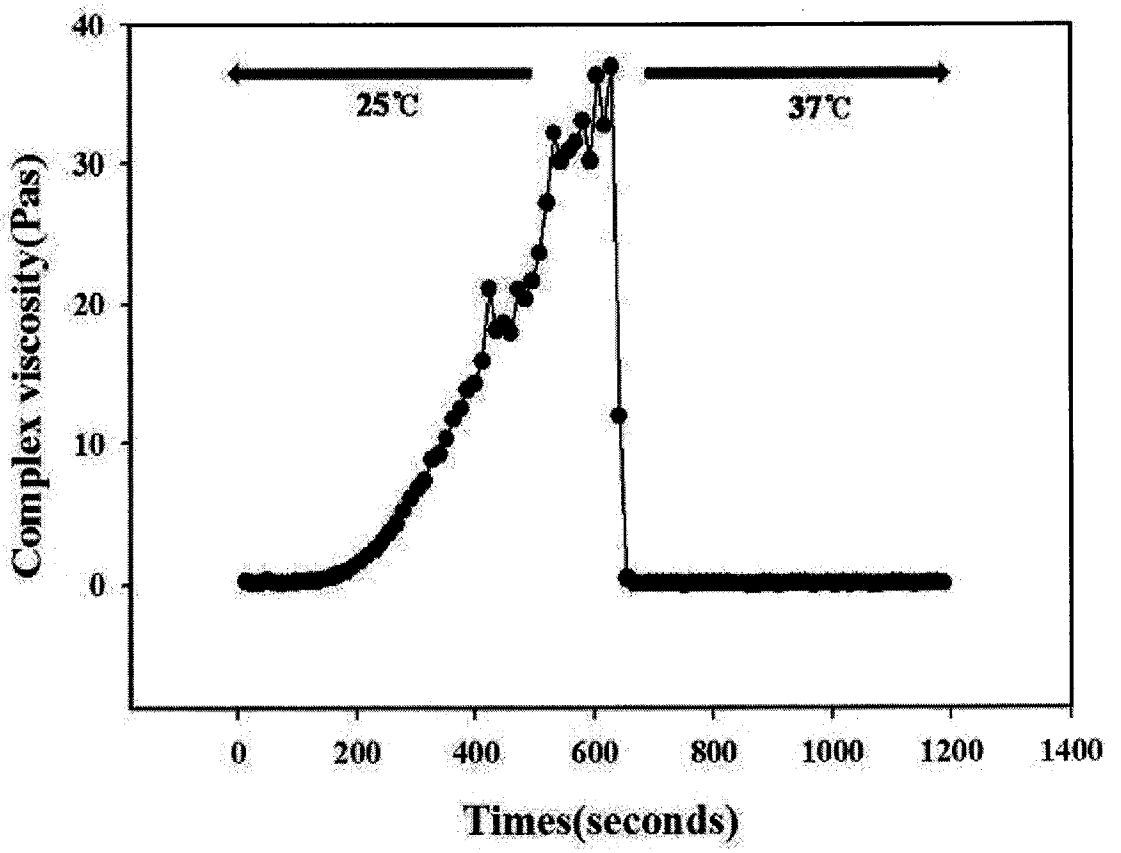

40
30
20
10
0
Complex viscosity(Pas)
25℃
37℃
0
200
400
600
800
1000
1200
1400
Times(seconds)

FIG. 4

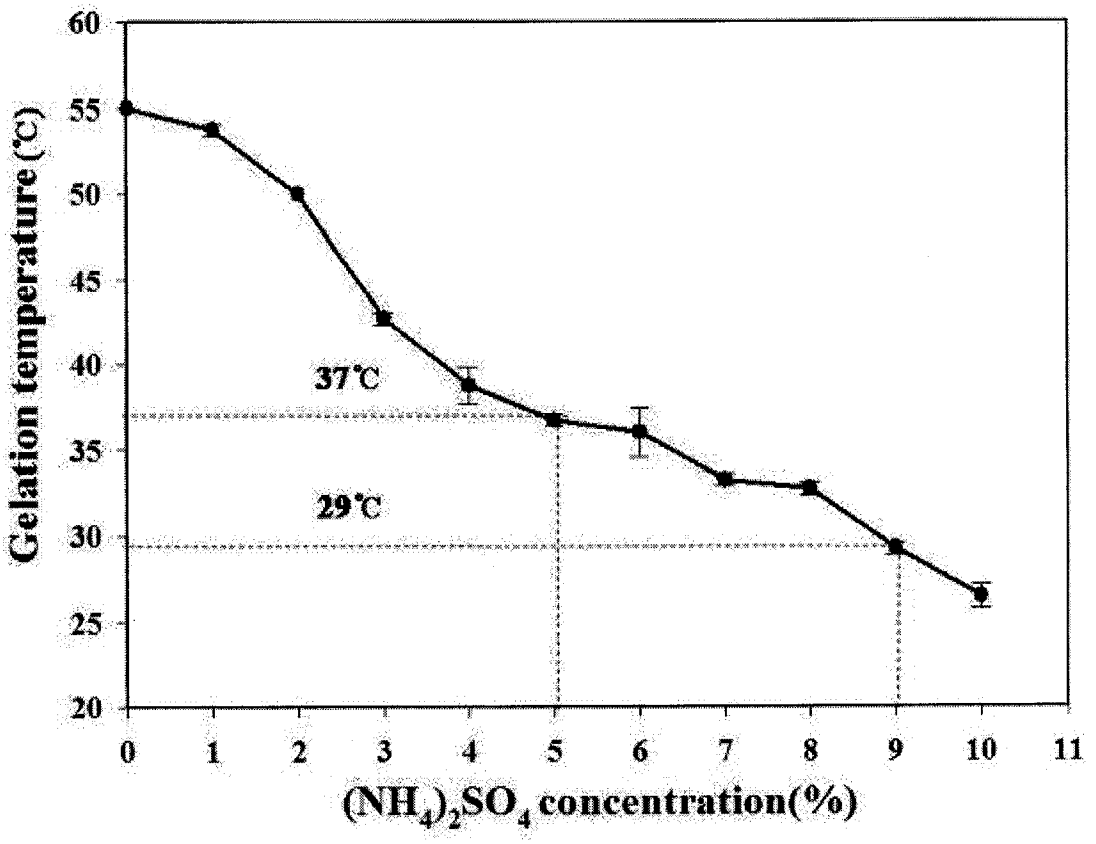

Gelation temperature (℃)
60
55
50
45
40
35
30
25
20
37℃
29℃
0
1
2
3
4
5
6
7
8
9
10
11
(NH4)2SO4 concentration(%)

FIG. 5

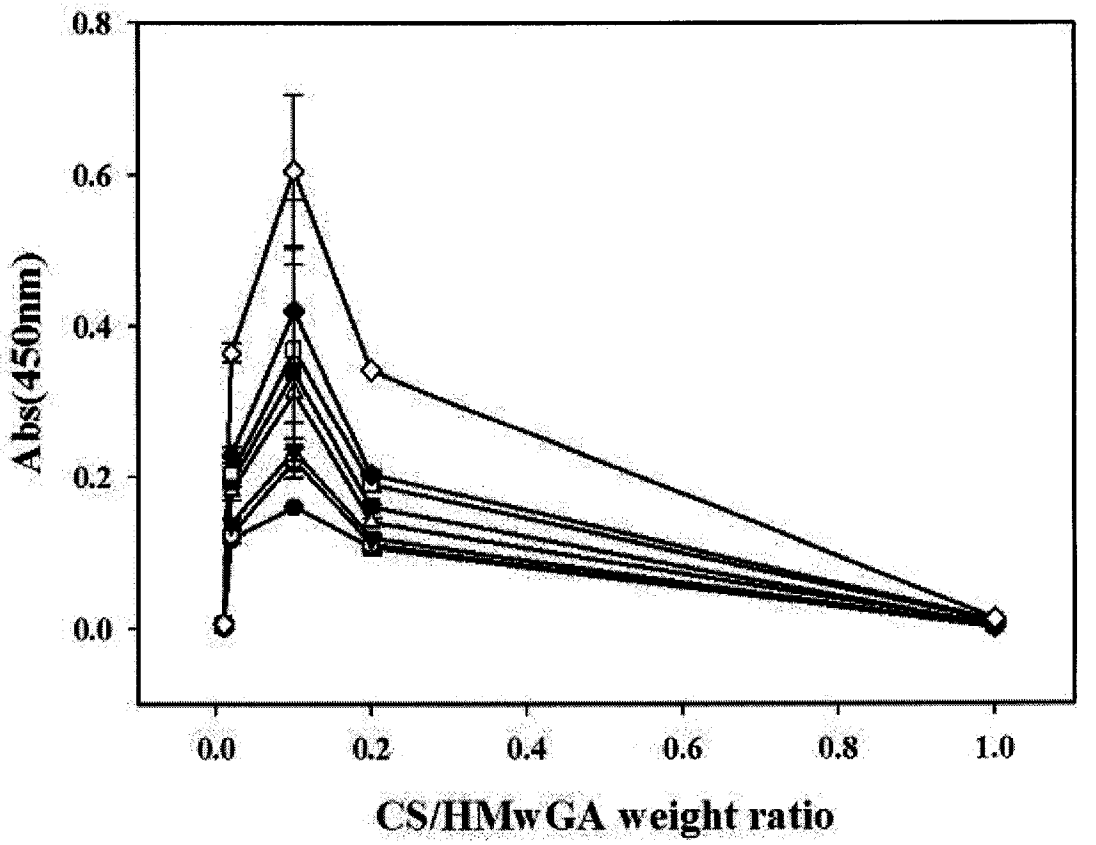


FIG. 6

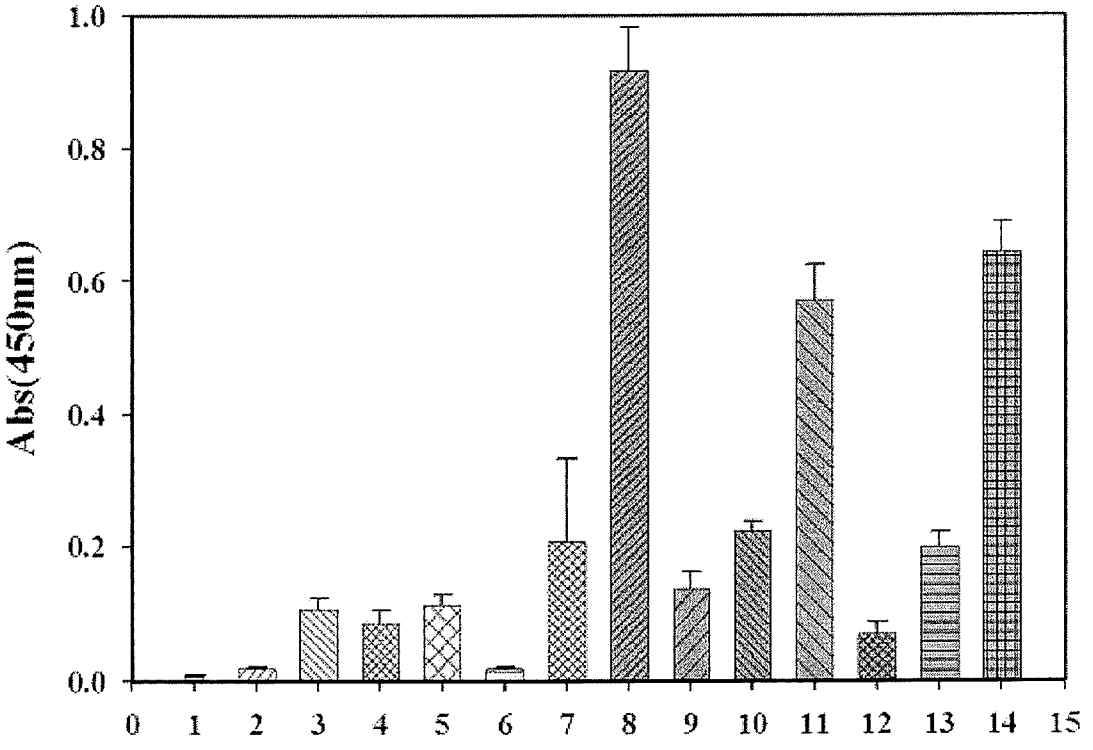


FIG. 7

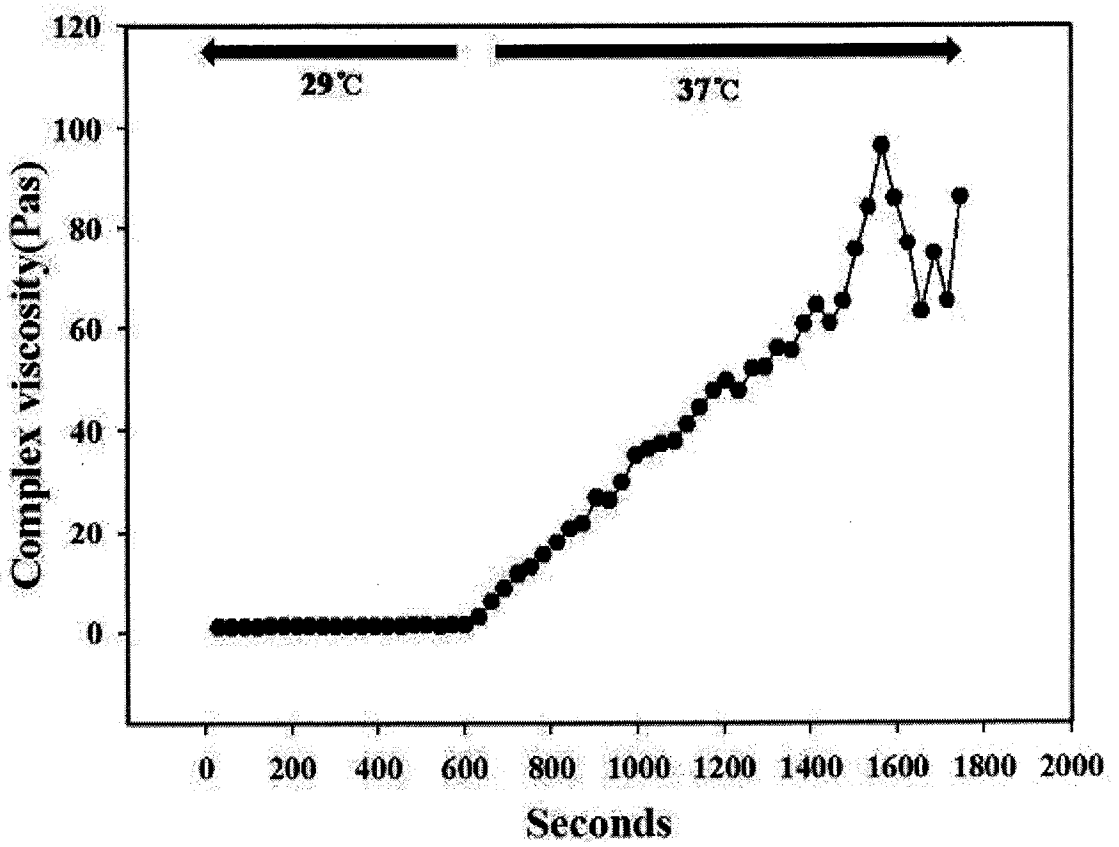

29℃
37℃
Complex viscosity(Pas)
120
100
80
60
40
20
0
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Seconds

FIG. 8

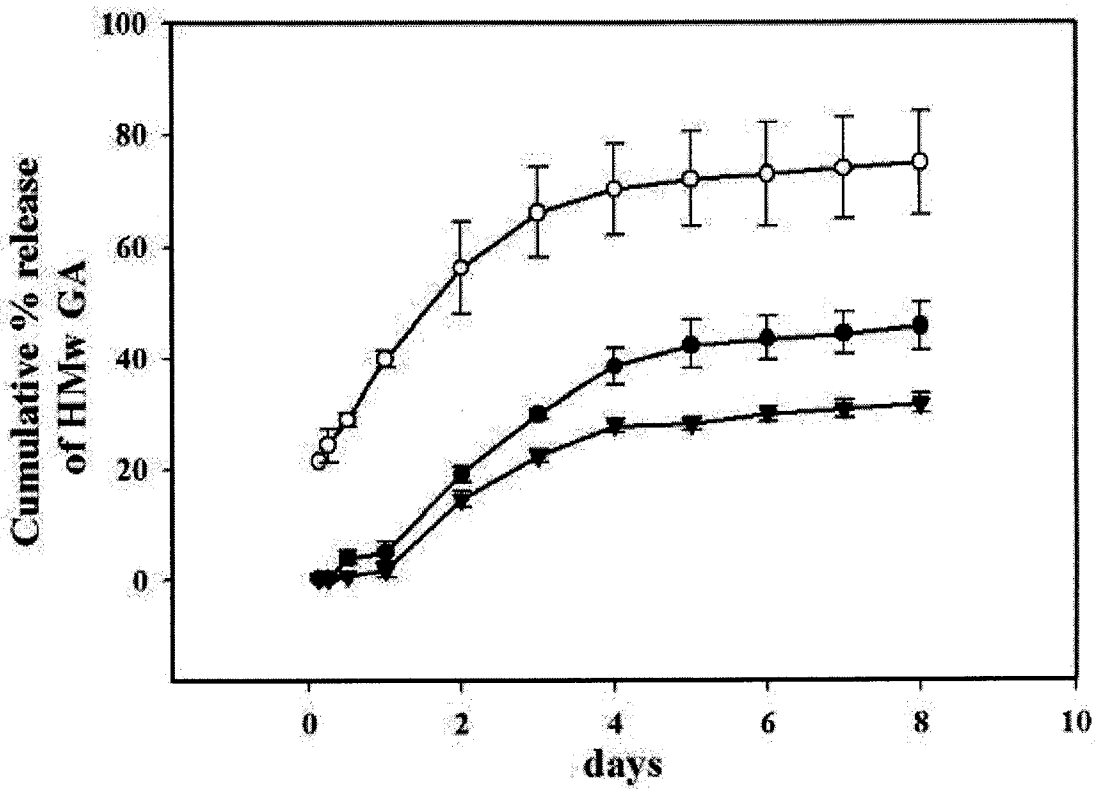


FIG. 9

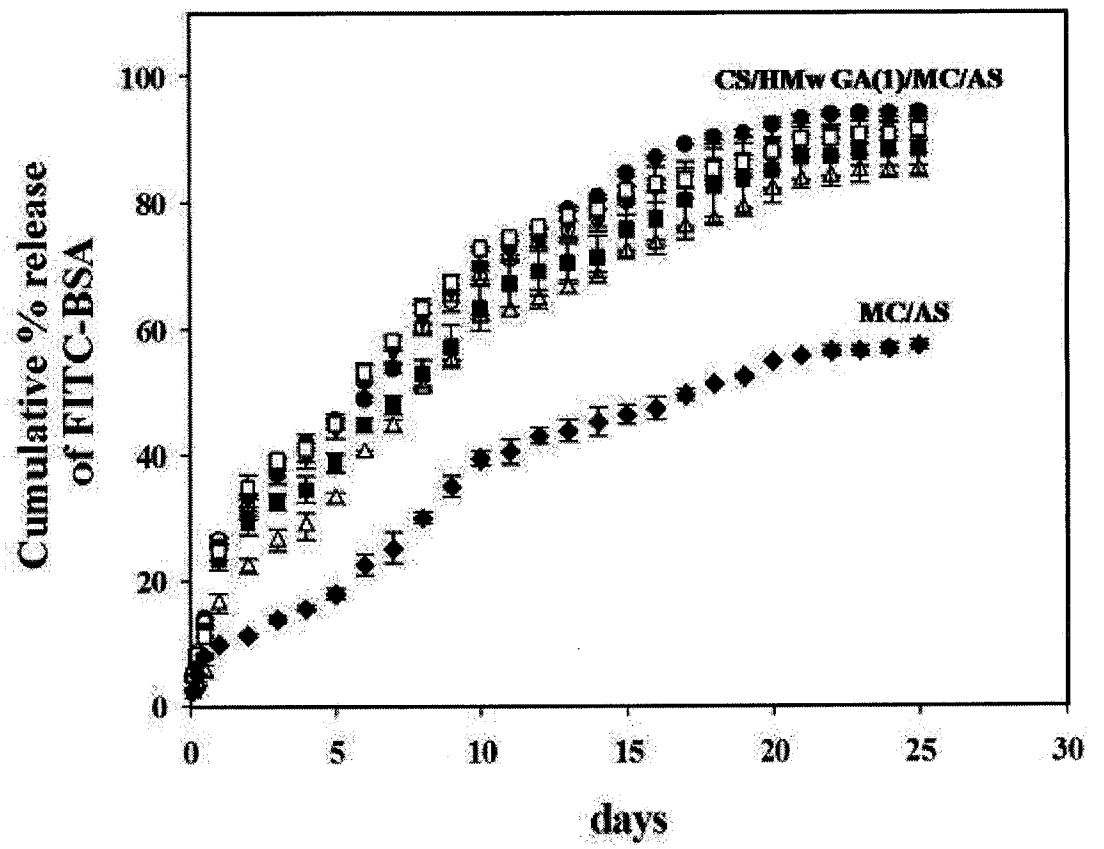
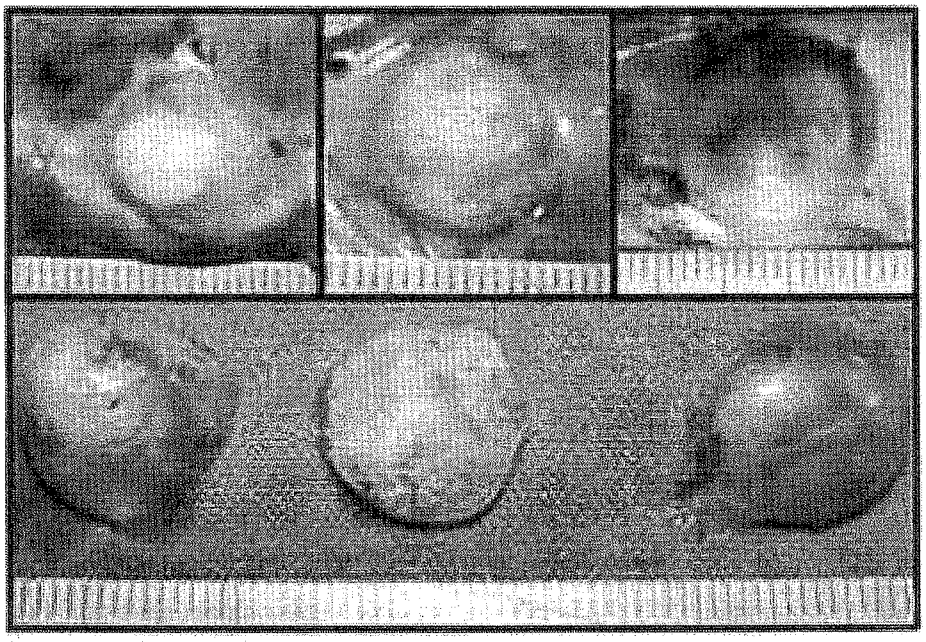

Cumulative % release of FITC-BSA
100
80
60
40
20
0
0
5
10
15
20
25
30
days
CS/HMw GA(1)/MC/AS
MC/AS

FIG. 10



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2007/005656

A. CLASSIFICATION OF SUBJECT MATTER

A61K 9/22(2006.01)i, A61K 9/50(2006.01)i, A61K 47/48(2006.01)i

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 : as above

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)

EKIPASS(KIPO internal), CAS(ON LINE), PUBMED

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2006/023207 A2 (THE UNITED STATES OF AMERICA as represented by THE SECRETARY OF HEALTH AND HUMAN SERVICES, NIH) 02 March 2006 See the abstract; paragraphs [0002], [0004]-[0006], [0014]-[0022]; example 1; claims 1-22.	1-15
A	US 5759582 A (THE JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE) 02 June 1998 See the column 2, lines 23-53; column 3, lines 19-33; column 6, lines 4-17.	1-15
A	US 6475995 B1 (THE JOHNS HOPKINS UNIVERSITY) 05 November 2002 See the column 4, lines 19-25; column 6, line 61-column 7, line 40.	1-15
A	US 6410517 B1 (JOHNS HOPKINS UNIVERSITY) 25 June 2002 See column 3, lines 38-41; column 6, lines 29-59; figure 1.	1-15

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

☒ See patent family annex.

* 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 citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
02 MAY 2008 (02.05.2008)	**02 MAY 2008 (02.05.2008)**

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

HAN, Jung Hee

Telephone No. 82-42-481-5604

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2007/005656

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006023207 A2	02.03.2006	US 20080075778 A1	27.03.2008
US 05759582 A	02.06.1998	None	
US 6475995 B1	05.11.2002	None	
US 6410517 B1	25.06.2002	None	