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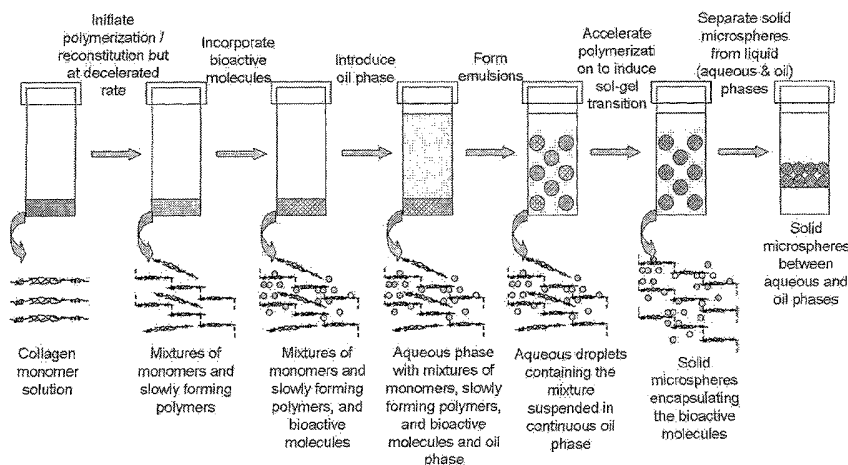


Figure 1

(57) Abstract: A method of manufacture of ECM microparticles incorporating bioactive molecules for drug delivery has been developed, using a modified emulsification method or a water-in-oil-phase-separation method. The microspheres are photochemically crosslinked to control the release of the bioactive molecules for better drug delivery usage without compromising the biocompatibility of the crosslinked structures. The method uses mild fabrication conditions and simple processes, no toxic chemical crosslinking reagent, which may cause cytotoxicity and calcification after implantation, no organic solvents, which may reduce drug availability and bioactivity, and no vigorous stirring action, which may fragmentize material with poor shape and mechanical stability and thus destabilize the emulsion. The resulting microparticles or microspheres are of controlled size, controlled release, highly biocompatible, and useful for drug delivery as well as cell culture.

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COLLAGEN-BASED MICROSPHERES AND METHODS OF PREPARATION AND USE THEREOF

FIELD OF THE INVENTION

5 The present application is generally related to microparticles formed of extracellular matrix materials that provide controlled release and desirable mechanical properties, and methods of manufacture and use thereof.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims benefit of and priority to U.S.S.N.
10 60/948,336 filed on July 6, 2007 and is a continuation-in-part of U.S.S.N. 11/225,108 filed on September 14, 2005, which claims benefit of and priority to U.S.S.N. 60/609,600 filed on September 14, 2004. The disclosures in the applications listed above are herein incorporated by reference.

BACKGROUND OF THE INVENTION

15 Microsphere-based drug delivery systems are advantageous because of their injectability and versatility in controlling the release patterns of the loaded drugs (Sinha and Trehan, *J. Control. Release*, 90(3):261-80 (2003)). This reduces invasiveness of multiple injections. Biocompatibility and biodegradability are necessary criteria for selecting the drug carriers. Both
20 synthetic polymers such as polylactic acids and polyglycolic acids and natural polymers such as chitosan and alginates can be used for making microspheres for drug delivery (Sinha and Trehan, *J. Control. Release*, 90(3):261-80 (2003)). Poly-lactic-glycolic acid (PLGA) microspheres dominate this field (Pean, et al., *J. Control. Release*, 56(1-3):175-87 (1998);
25 Sinha and Trehan, *J. Control. Release*, 90(3):261-80 (2003); Jiang, et al., *Adv. Drug Deliv. Rev.*, 57(3):391-410 (2005)) because it has been used for

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years as a suture material. However, as a polyester, PLGA has inevitable intrinsic shortcomings (Sinha and Trehan, *J. Control. Release*, 90(3):261-80 (2003)) such as low protein polymer compatibility due to the limited solubility and stability of protein in the hydrophobic PLGA matrix, and the extreme acidity of the degradation products at local injury site, which can damage cells and denature proteins. Typically fabricated by a double-emulsion technique, PLGA microspheres often show low encapsulation efficiency and poor retention of bioactivity of the encapsulated protein. Previous reports have demonstrated large burst effect of nerve growth factor (“NGF”) release and loss of bioactivity as early as 48 hours (Pean, et al., *J. Control. Release*, 56(1-3):175-87 (1998); Hadlock, et al., *J. Reconstr. Microsurg.*, 19(3):179-84; discussion 185-6 (2003)). Apart from instability of the protein drugs, high initial burst and incomplete release also affect the efficiency of microsphere-based drug delivery systems (Yeo and Park, *Arch. Pharm. Res.*, 27(1):1-12 (2004)).

Strategies aiming to improve the initial rapid loss using methods enhancing the protein distribution throughout the polymer matrix (Fu, et al., *J. Pharm. Sci.*, 92(8):1582-91 (2003)) have been reported. Polymerization, emulsification, spray drying and solvent extraction or combinations of these processes are commonly used methods for preparation of polymeric microspheres (Freiberg and Zhu, *Int. J. Pharm.*, 282(1-2):1-18 (2004)). US Patent No. 6,630,156 to Seo, et al. discloses a method for producing polymer microspheres incorporating physiologically active molecules by emulsification followed by solvent extraction. These methods involve the usage of organic solvents, emulsifying stabilizer and vigorous stirring. These present chemical and mechanical stresses, which can exert damaging

effects on the conformational and biological integrity of many drugs in particular proteins (Yeo and Park, *Arch. Pharm. Res.*, 27(1):1-12 (2004)). Moreover, the acidic and hydrophobic microenvironment within the degrading polymers can further damage the loaded drugs (Freiberg and Zhu, 5 *Int. J. Pharm.*, 282(1-2):1-18 (2004)).

Natural extracellular matrix such as collagen has excellent biocompatibility and negligible immunogenicity (Sano, et al., *Adv. Drug Deliv. Rev.*, 31(3):247-266 (1998); Lee, et al. 2001), and excellent protein compatibility. Therefore, they are excellent candidates for protein delivery 10 devices. These materials provide the natural extracellular milieu that stabilize proteins and potentiate or augment the activity of protein drugs such as growth factors (Lee, et al., *Int. J. Pharm.*, 221(1-2):1-22 (2001); Jones, et al., *J. Physiol.*, 533(1):83-9 (2001); Milev, et al., *J. Biol. Chem.*, 273(34):21439-42 (1998)). Moreover, degradation of these materials results 15 in naturally occurring monomers at neutral pH that do not generate local injury or inflammation. Furthermore, these materials facilitate cell adhesion, attachment and growth that may help in efficiently delivering the signals regulating cell activities. However, their development as drug delivery devices have been overshadowed by advances in synthetic polymers due to 20 the poor dimensional and mechanical stability, and rapid swelling properties of these natural extracellular matrix biomaterials (Yannas, et al. IV. Natural materials. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE, editors. *Biomaterials Sciences - An introduction to materials in medicine*. California: Academic Press, 1996: 84-93). This is because most microsphere fabrication 25 method requires vigorous mixing that may fragment these materials (Freiberg and Zhu, *Int. J. Pharm.*, 282(1-2):1-18 (2004)). Therefore, it is

almost impossible to fabricate microspheres using these materials unless chemical crosslinking has been used. However, toxicity associated with the residue chemical crosslinking agent prevents its use in drug delivery (Sinha and Trehan, *J. Control. Release*, 90(3):261-80 (2003)). US Patent
5 Application 20060222680 by Yang and Mark discloses a method of preparing chemically crosslinked collagen microspheres. Chemical crosslinking using glutaraldehyde is efficient in crosslinking the polymers with enhanced mechanical and shape stability. However, it compromises the biocompatibility of the crosslinked structures because the toxic residual
10 chemicals and degradation products induce cytotoxicity and calcification (Simmons, et al., *Biotechnol. Appl. Biochem.*, 17(Pt 1):23-9 (1993)).

Drug release from a matrix carrier is controlled by either diffusion or degradation or in many cases combinations of two. Many formulation parameters, including the drug, matrix and environmental factors affect the
15 initial burst and the rate of release (Yeo and Park, *Arch. Pharm. Res.*, 27(1):1-12 (2004)). Drug factors such as surface charge, hydrophobicity, loading and solubility in the continuous phase of the drug may affect the interaction between the drug and the matrix and thus the initial burst and release rate. Matrix factors such as the hydrophilicity, concentration,
20 porosity, density, mesh size and swelling properties of the matrix also affect the interaction between the drug and the matrix and thus the initial burst and release rate.

It is therefore an object of the present invention to provide microparticles formed of ECM materials that provide controlled release of
25 bioactive materials and desirable mechanical properties, and methods of manufacture and use thereof.

It is another object of the invention to provide microparticles formed of ECM materials that are highly compatible with bioactive materials and particularly peptides, polypeptides and proteins.

It is a further object of the present invention to provide methods of
5 manufacture that are simple, mild, and non-toxic, without vigorous stirring or organic solvents.

SUMMARY OF THE INVENTION

A method of manufacture of ECM microparticles incorporating bioactive molecules for drug delivery and/or for tissue engineering has been
10 developed, using a modified emulsification method or a water-in-oil-phase-separation method. The microspheres are photochemically crosslinked to control the release of the bioactive molecules for better drug delivery usage and to form structure without compromising the biocompatibility of the crosslinked structures. The method uses mild fabrication conditions and
15 simple processes, no toxic chemical crosslinking reagents, which may cause cytotoxicity and calcification after implantation, no organic solvents, which may reduce drug availability and bioactivity, and no vigorous stirring action, which may fragmentize material with poor shape and mechanical stability and thus destabilize the emulsion. The resulting microparticles or
20 microspheres are of controlled size, controlled release, highly biocompatible, and useful for drug delivery as well as cell culture.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a flow chart showing the process of preparing the microspheres using the methods disclosed herein.

25 **Figure 2** is a flow chart showing the process of photochemical crosslinking of the microspheres.

Figure 3 is a line graph showing the size distribution of microspheres, with 1% TWEEN@20 (-◆-) or without TWEEN@20 (-■-). Size distribution is graphed as proportion of microspheres (percent) as a function of size (microns).

5 **Figure 4** is a line graph showing controlled release and reduced burst effect of BSA (percentage of encapsulated BSA) from microspheres with (-■-) or without (-◆-) 0.01% photocrosslinker ("PC") Rose Bengal ("RB"). The rate of release of BSA from the particles is graphed as encapsulated BSA released (percent) as a function of time (hours).

10 **Figure 5** is a line graph of the dose dependence of RB on release patterns of BSA. Tested concentrations of RB are 0 (-◆-), 0.01 (-▲-), 0.001 (-x-), 0.0001 (-*-), 0.00001 (-■-), and 0.000001 (-+-) percent. Release of BSA from the microparticles is graphed as concentration of BSA/microgram over time (hours).

15 **Figure 6** is a line graph showing controlled release and reduced burst effect of microspheres with and without PC. Tested concentrations of RB are 0 (-◆-), 0.01 (-■-), 0.001 (-▲-), and 0.0001 (-x-) percent. BSA release from the microparticles is graphed as amount of BSA released (micrograms) as a
20 function of time (hours).

Figure 7 is a line graph showing a standard curve of nerve growth factor (NGF) bioactivity as a function of concentration of NGF (ng/ml). NGF bioactivity was measured as the proportion of PC12 cells with neurite

outgrowth longer than one body length using 1.5626, 3.125, 6.25, 12.5 and 25 ng/ml of NGF. Over 100 cells were counted in each group.

Figure 8 is a bar graph showing control of encapsulation efficiency (percent) as a function of the amount of protein loaded, for 1000, 500, 100, 5 and 75 micrograms.

Figure 9 is a bar graph showing the percentage distribution of NGF in collagen microspheres with and without photochemical crosslinking. (n=5)

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

10 Extracellular matrix (“ECM”) as used herein refers to any material produced by cells and secreted into the surrounding medium, but is usually applied to the non-cellular portion of animal tissues. The ECM of connective tissue is particularly extensive and the properties of the ECM determine the properties of the tissue. In broad terms there are three major
15 components: fibrous elements (particularly collagen, elastin, or reticulin), link proteins (e.g. fibronectin, laminin), and space-filling molecules (usually glycosaminoglycans).

“Microparticles” as used herein includes microspheres (generally spherical in shape) and microcapsules (generally spherical in shape with a
20 hollow centre), as well as particles of irregular shape, and are typically in the range of between 0.5 to less than 1000 microns in diameter.

II. Methods of Manufacture

The method of preparing microspheres of collagen and composites of collagen and other extracellular matrix components, as depicted in Figure 1, comprises the steps of:

- 5 (1) providing a solution of collagen monomers or mixture of collagen monomers and other natural extracellular matrix components;
- (2) initializing the sol-gel transition of collagen or precipitation of collagen and other matrix components;
- (3) decelerating the sol-gel transition or the precipitation process for appropriate period of time;
- 10 (4) incorporating one or more bioactive molecules into the aqueous gelling mixture at appropriate time;
- (5) mixing the aqueous phase with an oil phase with appropriate viscosity for appropriate time at appropriate agitation rate with or without the presence of surfactant;
- 15 (6) accelerating the sol-gel transition or the precipitation process after appropriate period of time;
- (7) separating the solid microspheres from the liquid phase which consists of both the oil phase and the aqueous phase by methods such as centrifuging the mixture at appropriate speed.

20 Although described with reference to microparticles, additional structures can be formed, and/or the microparticles can be formed into additional structures as coatings, encapsulated or incorporated into other materials that act as binders, for example, for use as tissue engineering matrices.

25 A. Materials

1. ECM Materials

Natural extracellular matrix components include, but are not limited to, collagen of different phenotypes such as type I, II and III, denatured collagen gelatin, proteoglycans, hyaluronic acid, elastin, both extracted from naturally occurring sources such as human and animal tissues or synthesized. These can be obtained using published procedures, or purchased from any of several suppliers. Some extracellular matrix components such as collagen, gelatin and hyaluronic acid can be induced to undergo sol-gel transition under certain specific conditions while other natural extracellular matrix components such as collagen and GAGs (glycosaminoglycans) precipitate with each other at other specific conditions.

In a preferred embodiment, the extracellular matrix is collagen type I, type II, type III, or mixtures thereof. This may be of human or animal or synthetic origin. In one embodiment, collagen is from rat tail, bovine Achilles tendon, porcine skin, or human placenta and from different fractions of extracted collagen such as acid-soluble fraction, pepsin digested fraction, and insoluble fraction. In another embodiment, proteoglycan is from shark cartilage. The collagen monomers can be from fractions of collagen extracted from animal sources, such as the acid-soluble fraction, pepsin digested fraction, or insoluble fraction.

2. Bioactive Materials

Any therapeutic, prophylactic or diagnostic material can be incorporated into the microspheres. These may be proteins or peptides, saccharides or polysaccharides, lipids or conjugates or complexes thereof, nucleic acids such as DNA, RNA or complexes thereof, or other inorganic or organic molecules, for example, antibiotics, chemotherapy drugs, etc.

The microspheres are manufactured using mild fabrication conditions that are particularly suitable for encapsulating proteinaceous bioactive agents. In one embodiment, the therapeutic, prophylactic or diagnostic material that is encapsulated is a peptide, polypeptide or protein. Preferred materials include growth factors such as but not limited to cytokines and growth factors, for example, nerve growth factor (NGF), basic fibroblast growth factor (bFGF), platelet-derived growth factors (PDGF), transforming growth factors beta (TGF- β), and insulin growth factor I (IGF-I). These factors are usually water soluble and will be mixed with the gelling mixture thoroughly so that any droplets formed during emulsification will contain evenly distributed factors. Molecules stabilizing these bioactive factors such as bovine serum albumin ("BSA") or human serum albumin ("HSA") for NGF can also be included.

3. Oils, Surfactants and Other Emulsifying Agents

The oil phase typically will include vegetable oils such as olive oil or corn oil, organic oils such as paraffin oil, synthetic oils such as silicone oil or mixtures of different oils, depending on the viscosity of the gelling mixture, for example, paraffin oil at a volume ratio between 1:1 to 1:100, preferably between 1:6 and 1:10.

The solution should preferably have a viscosity in the range of between 1 and 1000 mPascal second, most preferably 20-100 mPas. The volume ratio referred to herein is the ratio of volume of the ECM component (collagen)-bioactive protein mixture to the volume of oil.

A surfactant may be added to either or both the aqueous solution or the oil. A preferred surfactant is TWEEN® 20 or 180, SDS, or TRITON®

X-100, most preferably TWEEN®20, added to 0.1, 1, 10%, 20% preferably 10% of liquid mixture.

B. Reaction Conditions

1. Providing a solution of collagen monomers or mixture 5 of collagen monomers and other natural extracellular matrix components.

The ECM material is provided as an aqueous solution of monomer, preferably in a range of 0.5-30 mg/ml, more preferably 7 mg/ml. The pH of acid soluble collagen is around 3. No dispersant or buffer is typically needed.
10 An aqueous collagen solution typically is adjusted to the acid pH with an acidic solution such as 0.02N acetic acid.

2. Initializing the sol-gel transition of collagen or precipitation of collagen and other matrix components.

The sol-gel transition or the precipitation process is usually initiated
15 by a chemical and/or physical reaction. In one embodiment, collagen monomers are initiated to polymerize or reconstitute to native fibrils by raising the pH such as addition of sodium hydroxide or sodium bicarbonate, the ionic strength or the temperature. In another embodiment, collagen monomers are mixed with GAGs and precipitation is initiated by mechanical
20 actions such as stirring, vortexing, agitating and rotating. In another embodiment, sol-gel transition of composite of acid-soluble collagen type I and II is initiated by raising the pH of the mixture by an alkali such as sodium hydroxide or sodium bicarbonate or ammonium hydroxide, preferably to a pH between 4 and 14, most preferably between 8 and 14. In
25 another embodiment, collagen monomers, or collagen monomers mixed with

other ECM components, are initiated to polymerize or reconstitute to native fibrils by increasing the temperature of the solution.

3. Decelerating the sol-gel transition or the precipitation process for appropriate period of time.

5 These natural extracellular matrix components will solidify during polymerization, sol-gel transition and precipitation until the completion of the process or until reaching the equilibrium after certain period of time. Before these components are completely solidified, the mixture is subjected to conditions at which the polymerization or the precipitation process is
10 decelerated or even temporarily inhibited by controlling the same or another parameter. This step is for incorporation of the bioactive molecules into the mixture and for formation of emulsions.

 Methods for decelerating the process include keeping the temperature as low as possible, such as between 2-25° C, most preferably 4° C or lower
15 but above 0° C. Other methods include adjusting the pH to lower than 7 such as 4, or lowering the ionic strength of the solution, such as by using 0.1X PBS.

 In one embodiment, the collagen gelling mixture is subjected to an environment of lower temperature such as 4° C. In another embodiment, the
20 collagen and GAGs mixture is subjected to an environment without mechanical disturbance. In another embodiment, the sol-gel transition of the matrix components is decelerated at 0, 1, 10 or 60 seconds, 3, 5, 10 or 60 minutes, 2, 4, 8 or 24 hours, or 2, 4, 7 or 14 days, throughout other steps before resuming the sol-gel transition. In a preferred embodiment, the sol-
25 gel transition is decelerated at 15 minutes.

4. Incorporating one or more bioactive molecules into the aqueous gelling mixture at appropriate time.

Single or multiple bioactive molecules can be incorporated into the mixture, either as a liquid or as a powder. Bioactive molecules are added to a therapeutically effective concentration. In many cases, such as with most protein growth factors, these are effective at pictogram to nanogram levels. Bioactive agents may also be incorporated in the milligram level. These will typically uniformly disperse, especially if the molecules are water soluble.

5. Mixing the aqueous phase with an oil phase with appropriate viscosity for an appropriate time at an appropriate agitation rate with or without the presence of surfactant.

Emulsions will be formed between the gelling or precipitating mixture of natural extracellular matrix components and an oil phase by methods including, but are not limited to, shaking, agitating, mixing, stirring, and vortexing, without fragmentizing the gelling mixture and disturbing the sol-gel transition process, preferably with a loading device, at appropriate volume ratios, with appropriate viscosity, at appropriate agitation speed for appropriate period of time. The viscosity will typically be between 1-1000 mPas (the unit milliPascal second), preferably 20-100 mPas, with an agitation speed between 800-10,000 rpm, preferably 2000 rpm, for a period of time between 1 second to 24 hours, preferably 10 seconds, with a volume ratio of the aqueous protein mixture to oil between 1:1 to 1:100, preferably 1:10.

In one embodiment, an ECM gelling mixture is mixed with an oil phase such as olive oil, silicon oil, corn oil, or mixtures of different oil, depending on the viscosity of the gelling mixture. The oil phase typically

will include vegetable oils such as olive oil or corn oil, organic oils such as paraffin oil, synthetic oils such as silicone oil or mixtures of different oils, depending on the viscosity of the gelling mixture, for example, paraffin oil at a volume ratio between 1:1 to 1:100, preferably between 1:6 and 1:10. The preferred material is paraffin oil at a volume ratio between 1:1 to 1:100, preferably 1:10, for a period of time such as 1, 10, 20, 30, 40, 50, or 60 seconds, 1, 2, 5, 10, or 60 minutes, or 2, 4, 8, or 24 hours. The period of time is determined by the rate of sol-gel transition, total volume of the collagen protein mixture and the oil, viscosities of collagen protein mixture and the oil phase, the power and frequency of the mechanical mixing machine, the size and mass of the containers holding the mixtures, temperature, etc. The examples demonstrate the ranges based on the maximal power of a custom-made mixing machine in a 10 ml plastic container, 7 mg/ml collagen with 25 mg BSA, volume ratio of 1:6 for collagen mixture to oil ratio, at 25° C. In a preferred embodiment, the material is mixed for 10 seconds, at one fourth of the maximal agitation speed of this custom-made mixing device. If the speed is too low, it will not provide sufficient energy to emulsify the mixture. If the speed is too fast, phase inversion will occur so that oil droplets will be trapped within the droplets of collagen gelling mixture and collagen fragments will be obtained. If the time for emulsification is too short, the material will coalesce to form microspheres with large diameter size. If the time for emulsification is too long, phase inversion and fragmentization will occur.

In order to further stabilize the emulsions formed, surfactants, including but are not limited to TWEEN® 20 can be introduced during this process, at a concentration of 1, 10, 20% preferably 1%.

6. Accelerating the sol-gel transition or the precipitation process after an appropriate period of time.

Immediately after the emulsion formation, the emulsions will be subjected to an environment accelerating or resuming the sol-gel transition or the precipitation process at a maximal rate for a period of time. This is to solidify the microdroplets in the emulsions so as to encapsulate the incorporated bioactive molecules within the homogenous solid mass of the microspheres. This also prevents coalescence so as to reduce the microsphere size. The same process parameters are manipulated.

10 In one embodiment, collagen emulsions are subjected to an environment with raised temperature at 12° C, 25° C, 37° C, preferably 37° C, for a period of time such as 10, 30 or 60 seconds, 2, 5, 10, 30 or 60 minutes, 2, 4, 8 or 24 hours, or 2, 4 or 7 days, preferably 45 minutes, until the sol-gel transition is completed or reached equilibrium thus forming solidified microspheres, depending on the properties of the sol-gel transition of the matrix component. In another embodiment, the rate of gelation of the collagen microspheres is controlled by varying the concentration of a phosphate buffered saline ("PBS", 0.01 M phosphate buffered saline, NaCl 0.138 M; KCl 0.0027 M.; pH 7.4, at 25° C.) buffer used at 1X, 5X and 10X preferably 5X. In another embodiment, the emulsions are subjected to an environment with pH raised to pH 14 by incubating the emulsions in an alkaline chamber filled with ammonia for solidification.

7. Separating the solid microspheres from the liquid phase which consists of both the oil phase and the aqueous phase.

25 The solidified microspheres are separated using standard techniques such as filtration, sedimentation, centrifugation, etc.

This method provides a simple process without involvement of organic solvents and vigorous mechanical and chemical stresses, which are known to reduce the bioactivity of drugs particularly protein drugs. The solidified microspheres are easily separated from both liquid phases, which
5 consist of both the aqueous and the oil phases. In one embodiment, the mixture is centrifuged at a speed such as 1, 10, 100, 1000 or 10000 rpm, preferably 4000 rpm, without fragmentizing the microspheres, for a period of time such as 1, 5, 10, 30 or 60 seconds, 2, 5, 10, 30 or 60 minutes, or 2, 4, 8 or 24 hours, preferably 10 minutes.

10 **III. Methods of Further Modifying Microparticles**

Microspheres are obtained having size distributions ranged from 0.05 to 1000 microns in diameter, preferably 50-100 microns. These microspheres have a smooth surface or a rough surface when dehydrated.

The initial burst effect can be reduced and the release pattern of the
15 bioactive molecules from the microspheres controlled by methods including, but not limited to, photochemical crosslinking, comprising the steps of (1) equilibrating the microspheres with a photosensitizing reagent in an appropriate dose; (2) rinsing the microspheres thoroughly to remove excess photosensitizer; and (3) irradiating the microspheres with a light source of
20 appropriate level of energy for a period of time.

A. Reagents

Photosensitizing reagents include chromophores able to be activated by photons at particular wavelengths, including, but not limited to, fluorescein, eosin, rose Bengal ("RB"), and methylene blue.
25 Photosensitizing reagents include RB, which has a spectrum of absorption up

to approximately 600 nm. In addition to the UV region, there are two absorption peaks at approximately 514 nm and 550 nm.

RB has high absorption efficiency and therefore is a very efficient photosensitizing reagent. Rose Bengal is a vital dye that has been used in clinical diagnostic for ophthalmologic diseases for decades (Lansche R.K.,
5 "Vital Staining in Normal Eyes and in Keratoconjunctivitis Sicca", Am. J. Ophthalmol. 60(3):520-5 (1965)). It is a safe reagent to use. Rose Bengal is a fluorescent photosensitizing reagent that is water soluble. However, aggregates may form at high concentrations such as >1% (w/v) and therefore
10 the preferred concentration of rose Bengal is ranged from 0.00001% to 0.01%. Rose Bengal solution is prepared in darkness using a solution such as distilled water or PBS and ethanol; preferably water.

B. Method of Crosslinking

1. Soaking microspheres in photosensitizer

15 The microspheres are immersed in a solution of photosensitizer for 1, 10, 30 or 60 seconds, 2, 5, 10, 30 or 60 minutes, or 2, 4, 8 or 24 hours, preferably 10 minutes. The concentration of the photosensitizing reagent ranges from 0.00001% to 1%, preferably 0.001%. Representative photosensitizing reagents include fluorescein, Rose Bengal, methylene blue,
20 eosin, and porphyrins.

Preferably the microspheres are into contact with the photosensitizing reagent for a period of time ranging from 5 seconds to 100 hours, preferably 10 minutes. Most preferably, excess reagent is removed from the microspheres by washing.

25 Photochemical crosslinking is a non-thermal, non-toxic and rapid technique to crosslink the collagen-based materials without compromising

the biocompatibility. This method is disclosed in US published Patent Application No. 20060099268. Photochemically crosslinked collagen has been demonstrated to have significantly enhanced physicochemical properties (Chan and So, *J. Biomed. Mater. Res. A*, 75(3):689-701 (2005),
5 Chan, et al., *Tissue Eng.*, (1): 73-85 (2007)). Apart from increased mechanical properties, thermal and chemical stability, the technique has been shown to dramatically reduce the pore size of collagen structures and the swelling rate, which alter the interactions between the collagen network and the loaded protein. These properties are important matrix parameters
10 affecting the initial burst and the release rate of the drug.

2. Exposing Microspheres to Light Source

The microspheres in water or an isotonic solution are brought to a light source, such as a UV source, a laser, LED or other source of visible light. The amount and power intensity of the light affect the extent of
15 crosslinking as it is proportional to the number of photons. Suitable light sources include an argon laser, at a wavelength of 514 nm, or multiline green laser. Other lasers which Rose Bengal absorbs can also be used. The argon laser can be continuous or pulsed. Laser will be delivered at an irradiance of 0.0001, 0.001, 0.01, 0.1, 1, or 10 W/cm², preferably 0.2W/cm². The duration
20 of irradiation is 1, 10, 100, 1000, or 10000 seconds, preferably 100 seconds. The total energy of light delivered to the microspheres is 0.5, 1, 5, 10, 100, or 1000 J, preferably 20 J.

The entire process can be performed under sterile conditions. During crosslinking, laser is delivered to the microspheres through a transparent
25 container.

C. Photocrosslinked Microspheres

Photochemically crosslinked microspheres have size distributions ranging from 0.05 to 1000 microns in diameter, preferably 50-100 microns. The photochemically crosslinked microspheres have smooth surface or rough surfaces when dehydrated.

5 Photochemical crosslinking reduces the release rate of incorporated bioactive molecules by both diffusion and degradation. Photochemical crosslinking strengthens the collagen fibrils that results in ultra-fine microstructures with micron-sized pores interconnected with nano-sized fibrous network in freeze-dried structures. This may reduce the rate of
10 diffusion and thus the release of the incorporated bioactive molecules from the liquid phase trapped among the fibrous network. Photochemical crosslinking also reduces the rate of disintegration of collagen structures in physiologically relevant conditions. Photochemical crosslinking also enhances retention of bioactive molecules with the matrix meshwork. This
15 reduces the amount and rate of release of the incorporated bioactive molecules in dehydrated samples upon rehydration. The crosslinked microspheres release the incorporated bioactive molecules with a significantly reduced initial burst and first order release kinetics. Photochemical crosslinking does not alter the mesh size of the hydrated
20 collagen fibril meshwork in the microsphere but may alter the interactions such as ionic, electrostatic and hydrophobic interactions, between the crosslinked collagen structures and the loaded proteins and thus hinder the release of proteins. The release can be sustained for a long term without compromising the bioactivity of the incorporated bioactive molecules.

25 Both uncrosslinked and photochemically crosslinked microspheres with incorporated bioactive molecules can be used to deliver the bioactive

molecules by direct injection into the injury sites systems including but are not limited to neurological and musculoskeletal systems.

D. Storage of Microspheres

The microspheres can be further processed and stored, for example, by dehydrating the microspheres rapidly or gradually by immersing in absolute alcohol or alcohol of increasing concentrations; freeze-drying, critical point drying, oven drying, air drying, acetone-drying, or dispersing in glycerol, or other conditions without denaturing both the matrix component of the microspheres and the incorporated bioactive molecules. The alcohol dehydration method extracts water from the microspheres by immersing in absolute alcohol for several rinses or a series of increasing concentration of alcohol, ranging from 10% to 100%, preferably 50% to 100%, each for a period of time ranging from 1 minute to 10 days, preferably 30 minutes. The microspheres can be stored as slurry or dried using dehydration gradient method.

E. Applications of Microspheres

The microspheres are useful for drug delivery and for three dimensional cell culture and implantation because these microspheres, unlike the chemically crosslinked ones, do not have toxicity.

The present invention will be further understood by reference to the following non-limiting examples.

Examples

Example 1. Preparation of collagen microspheres incorporated with BSA or myoglobin

Rattail collagen type I solution at a concentration of 7 mg/ml in 0.02N acetic acid was neutralized with 1N sodium hydroxide in the presence

of 10X phosphate buffered saline with a final concentration of 0.5X. The gelling mixture was placed inside an ice water bath at 4° C to slow down the polymerization rate for 1 minute. Sample proteins such as BSA and myoglobin can be incorporated. BSA (4mg) was added to the mixture and
5 thoroughly mixed well.

A mixture of olive oil and silicon oil at a ratio of 1:1 was laid down on the aqueous gelling mixture at a volume ratio of 6:1. A nonionic surfactant, Tween 20, was added to the aqueous phase before emulsification. The container of the mixture was placed on a mixing device. The mixture
10 was agitated at maximal speed (3000 rpm) for 30 seconds. The emulsions formed were then placed in a 37° C water bath to speed up the polymerization. The mixture was incubated for 30 minutes until equilibrium. The mixture was centrifuged briefly at 4000 rpm for 10 minutes to separate
15 the solid microspheres from the liquid phases including an aqueous and an oil phase. After discarding the oil and the liquid phase, the microspheres were rinsed twice and ready for injection or subsequent release experiments.

Example 2. Photochemical crosslinking of collagen microspheres

Microspheres obtained from procedures described in Example 1 were immersed in a solution of Rose Bengal (photocrosslinker, "PC") at a
20 concentration of 0.001% (w/v) in water for 10 minutes. Excess Rose Bengal was discarded and the microspheres were rinsed. The microspheres were resuspended in water and placed in a 4-well plate culture dish. An Argon laser at 514 nm was used to irradiate the microspheres at 0.02 W/cm² for 100
seconds. Immediately after the irradiation, the microspheres were rinsed in
25 water. The microspheres were then ready for injection or subsequent experiments.

Example 3. Dehydration of collagen microspheres

Microspheres were immersed in 100% alcohol for 30 minutes and the extraction of water from the microspheres was repeated for three times. A gradual increase in alcohol concentration was used to improve the
5 microsphere surface smoothness. The collagen microspheres were immersed in 50% v/v alcohol for 20 minutes, 70% alcohol for 30 minutes twice, 80% alcohol twice, 90% alcohol twice and 100% alcohol twice.

Example 4. Morphological analysis of microspheres

Microspheres were fixed in 0.25% glutaraldehyde for 4 hours at room
10 temperature and then dried by critical point drying. The microspheres were then thoroughly rinsed in large volume of water. The microspheres were dehydrated by critical point drying and mounted on the sample stage with carbon cement. The samples were sputtered with gold and analyzed using scanning electron microscopy (SEM). The results show that the
15 microspheres are much stronger and have smaller porosity when crosslinked.

Example 5. Characterization of microstructure of photochemically crosslinked collagen

Reconstituted collagen gel was photochemically crosslinked by varying the photosensitizing reagent and light dosage. Glutaraldehyde was
20 used as a positive control for chemical crosslinking. The treated structures were freeze-dried. Cross-sections of the collagen scaffolds were sputtered with gold for SEM analysis of the porous structures. This showed that both glutaraldehyde and photochemically crosslinked collagen scaffolds have fine microstructures with interconnected fibers with nanosized fibers and micro-
25 sized pores. In the control groups, only macrostructures with membrane like structures were found.

Tables 1-3 show the change in microstructures of collagen after photochemical crosslinking and the dose-dependence on photosensitizing reagent concentration and fluence. Table 4 shows the change in fiber size and mesh size after photochemical crosslinking and surfactant treatment.

5 Table 1 – Pore size distributions of collagen scaffolds.

Treatment groups	Pore size (microns) / Mean±SD	
	Long axis	Short axis
Control	366±61	224±44
Laser only	398±18	279±11
Dye only	267±12	170±10
Photochemical crosslinking (20J, 0.01% dye)	1.9±0.1	1.2±0.1
Glutaraldehyde (0.25%, 2hrs)	1.8±0.0	1.3±0.0

Table 2 – Pore size distributions of collagen scaffolds with different laser energy dosage and post-irradiation incubation time.

Duration of incubation	0hr		3hrs		24 hrs	
	Long axis	Short axis	Long axis	Short axis	Long axis	Short axis
0	No microstructure		No microstructure		No microstructure	
0.2	No microstructure		No microstructure		3.4±1.2	2.2±0.7
2	No microstructure		2.8±0.8	2.0±0.7	3.3±1.2	2.3±0.7
20	1.9±0.5	1.4±0.4	2.0±0.5	1.5±0.5	1.8±0.5	1.2±0.3

Table 3 – Pore size distributions of collagen scaffolds with different photosensitizer dosages.

	Long axis	Short axis
[RB]/ μM	Pore size (micron)	
0.1572	--	--
0.786	2.5+0.9	1.7+0.7
3.93	2.4+0.8	1.9+0.7
19.65	2.7+0.9	1.9+0.6
98.25	2.2+0.8	1.5+0.6

Table 4 – Fiber size, volume fraction and mesh size of the fibrous meshwork

5 in collagen microsphere of different treatment groups. (n=3)

		Fiber sizes (nm)	Volume fractions	Mesh sizes (nm)
Without PC	Without Tween 20	49.45 \pm 9.80	0.0079 \pm 0.0006	414.11 \pm 18.27
	With Tween 20	44.31 \pm 7.77	0.0086 \pm 0.0007	353.39 \pm 15.72
With PC	Without Tween 20	51.70 \pm 8.76	0.0094 \pm 0.0016	395.83 \pm 37.88
	With Tween 20	42.28 \pm 7.77	0.0088 \pm 0.0004	332.17 \pm 8.89

Example 6. BSA release pattern

25 mg of BSA was incorporated into 1 mg/ml of collagen microspheres as described in Example 1. Collagen microspheres were resuspended in 5 ml of 1X PBS in an Eppendoff tube and incubated in 37°C water-bath with regular agitation at 200 rpm. 2ml of the supernatant was removed with replacement at 0.5, 1, 2, 4, 8, 24 hours, 2, 4, 7, 14, and 35 days. The supernatant was diluted to the appropriate range for detection of the concentration of BSA using Bio-Rad protein assay kit.

Figures 4, 5 and 6 show that the photochemically crosslinked collagen microspheres have significantly reduced initial burst with an almost first order release kinetics of BSA. For the uncrosslinked microspheres, the initial burst effect resulted in a loss of almost 40% of the total amount loaded that significantly reduced the efficacy as drug delivery device in particular precious protein drugs. The release profile lasted for more than 35 days and the release is still linear in photochemically crosslinked microspheres.

Example 7. Retention of NGF bioactivity after photochemical crosslinking and surfactant treatment

2.5 μg NGF in 5 mg BSA was incorporated into collagen microspheres as described in Example 1. Photochemical crosslinking was conducted as described in Example 2. Another group uses 1% Tween 20 as surfactant. The microspheres were suspended in 1 ml medium and incubated in 37° C water bath for 1-4 days.

At the end of incubation, the supernatant was removed and used to incubate PC12 cells. PC12 cells were maintained in growth on 24-well plate in 81.5% F12K medium, supplemented with 15% Horse serum, 2.5% Fetal bovine serum and 1% PBS. The cells were cultured at 37° C in a water-saturated 5% CO₂, 95% air atmosphere and plated at a density of approximately 3,000 cells (in 800 μl) per well. After 1-3 days, the cells were fixed for visualization under the phase microscope. Cells with neurite outgrowth longer than one body length of the cell were counted and the percentage of PC12 cells with neurite outgrowth was recorded. The amount of PC12 cells with neurite growth was calibrated against a standard curve using known concentrations of NGF. The standard curve is shown in Figure 7. Part of the NGF released from the microspheres was incubated with PC12

cells while part of the sample was used for measurement of NGF concentration using ELISA. This allowed for a determination of the amount of released NGF that retained bioactivity.

The results demonstrated that NGF retained its bioactivity by stimulating PC12 differentiation and neurite growth as compared to the controls. Table 5 shows that the NGF released from microspheres treated with photochemical crosslinking or the surfactant retained almost all of their expected bioactivity, similar to those in the control.

10 Table 5 – Retention of bioactivity of encapsulated NGF.

	Quantity of NGF measured by ELISA (ng) (mean \pm SD)	Bioactivity of NGF measured by PC12 assay (ng) (mean \pm SD)
Control	28.67 \pm 4.26	25.48 \pm 11.63
Tween 20	6.31 \pm 0.73	5.42 \pm 7.91
PC	3.14 \pm 0.55	3.42 \pm 4.9

Example 8. Retention of bioactivity of NGF immobilized inside the collagen microspheres

In a separate experiment, NGF incorporated in the collagen microspheres was incubated at 37° C for 1, 2, 3 and 4 days. At the end of each time point, the supernatant was discarded and the microspheres were digested with bacterial collagenase at 200 U/ml for 5 hours. The digestion mixture was then diluted into the appropriate range. Part of the sample was used to incubate with PC12 cells while part of the sample was used for concentration measurement using ELISA kit.

The results showed that NGF retained in the collagen microspheres is still bioactive even after 4 days as it induced neurite growth in PC12 cells.

Example 9. Measurement of encapsulation efficiency

Collagen microspheres were formed as described in Example 1. The aqueous phase separated from the microspheres and the oil phase was pooled with the rinses of the microspheres. The pooled solution was mixed well by
5 vortexing and an aliquot of the washings was subjected to measurement of the incorporated BSA or myoglobin. The encapsulation efficiency was then calculated as follows:

$$\left[\frac{\text{total amount loaded} - \text{amount in the washings}}{\text{total amount loaded}} \right] \times 100\%.$$

10 The total amounts of protein loaded and the concentration of the collagen used in microsphere formation were varied to study their effects on the encapsulation efficiency. Figure 8 shows that the encapsulation efficiency decreases as the amount of protein being loaded increased. Figure 9 shows that the amount of NGF retained in the photochemically crosslinked
15 microspheres was higher than that in the uncrosslinked microspheres.

Example 10. Effects of surfactant

Collagen microspheres were prepared as described in Example 1 except that a non-ionic surfactant TWEEN® 20 was additionally used. After incorporating the bioactive molecule, different concentrations of TWEEN®
20 20 (1, 5 and 10%) were mixed with the aqueous phase before addition of the oil phase. The emulsion was formed as described in Example 1 and the microspheres were separated from the liquid phase by centrifugation. The microspheres formed in the presence of surfactant were analyzed for morphology and release pattern.

25 The results show that an increase in surfactant concentration results in a more stabilized emulsion as shown by less phase inversion and more

homogenous populations of the microspheres. Moreover, the size distribution of the microspheres has been shown shifted towards a smaller median value as the surfactant concentration increased.

Unless defined otherwise, all technical and scientific terms used
5 herein have the same meanings as commonly understood by one of skill in the art to which the disclosed invention belongs. Publications cited herein and the materials for which they are cited are specifically incorporated by reference.

Those skilled in the art will recognize, or be able to ascertain using no
10 more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

WHAT IS CLAIMED IS:

1. A method for preparing natural extracellular matrix-based microspheres, comprising
5 providing an aqueous solution of one or more natural extracellular matrix components;
initiating the sol-gel transition of the matrix components to form an aqueous gelling mixture;
decelerating the sol-gel transition process;
10 incorporating therapeutic, prophylactic or diagnostic molecules into the aqueous gelling mixture;
mixing the aqueous gelling mixture with an oil phase with agitation to form a water-in-oil emulsion;
accelerating the sol-gel transition process; and
15 separating the solid microspheres from the oil phase and the aqueous phase.
2. The method of claim 1 further comprising photochemically crosslinking the microspheres to reduce the initial burst effect or control the release of the molecules from the microspheres.
- 20 3. The method of claim 2 comprising
equilibrating the microspheres with photosensitizing reagent; and
irradiating the microspheres with a light source for a period of time effective to crosslink some or all of the extracellular matrix components.
4. The method of claim 1 further comprising dehydrating the
25 microspheres.

5. The method of claim 4 wherein the microspheres are dehydrated by immersion in an alcohol or glycerol.
6. The method of claim 1 wherein the natural extracellular matrix is selected from the group consisting of collagen, gelatin, proteoglycan, hyaluronic acid, and elastin.
7. The method of claim 1 wherein the extracellular matrix solution contains monomers or the soluble states of the extracellular matrix components.
8. The method of claim 6 wherein the extracellular matrix component is collagen type I, II, III or mixtures thereof.
9. The method of claim 1 wherein the sol-gel transition is initiated by polymerization, precipitation or aggregation.
10. The method of claim 9 wherein the sol-gel transition is initiated by controlling the pH, temperature or ionic strength depending on the sol-gel transition properties of the matrix component.
11. The method of claim 10 wherein the sol-gel transition of acid-soluble collagen is initiated by raising the pH or ionic strength or temperature such as 37° C.
12. The method of claim 1 wherein the sol-gel transition of the matrix components is decelerated after the sol-gel transition is initiated.
13. The method of claim 12 wherein the sol-gel transition of the matrix components is decelerated by controlling the pH, the temperature, the ionic strength, or the kinetic movement of the matrix components mixtures.
14. The method of claim 1 wherein the aqueous gelling mixture is mixed with the oil phase to form an emulsion without fragmentizing the gelling mixture and disturbing the sol-gel transition process.

15. The method of claim 1 wherein the sol-gel transition is accelerated after emulsion formation.

16. The method of claim 2 wherein the light source is a UV or visible light source operated at an irradiance variation of from 0.0001 W/cm² to 10 W/cm², preferably 0.2 W/cm², and

wherein the irradiation energy ranges from 0.0001 J to 10000 J, preferably 25 J, for a period of irradiation ranging from 3 seconds to 100 hours, preferably 60 seconds.

17. A population of microspheres formed by the method of claim 1.

18. The population of claim 17 wherein the microspheres have size distributions ranging from 5 to 2000 microns.

19. The population of claim 17 wherein the photochemically crosslinked microspheres release the incorporated molecules with significantly reduced initial burst effect as compared to non-photochemically crosslinked microspheres.

20. The population of claim 17 wherein the photochemically crosslinked microspheres release the incorporated bioactive molecules with significantly reduced rate as compared to non-photochemically crosslinked microspheres.

21. The population of claim 17 wherein the photochemically crosslinked microspheres release the incorporated molecules with first order release kinetics.

22. The population of claim 17 wherein the photochemically crosslinked microspheres retain more bioactive molecules in the matrix as compared to non-photochemically crosslinked microspheres.

23. The population of claim 17 wherein the incorporated bioactive molecules retain their bioactivities in photochemically crosslinked microspheres.

5 24. The population of claim 17 formed into structures for use in tissue engineering.

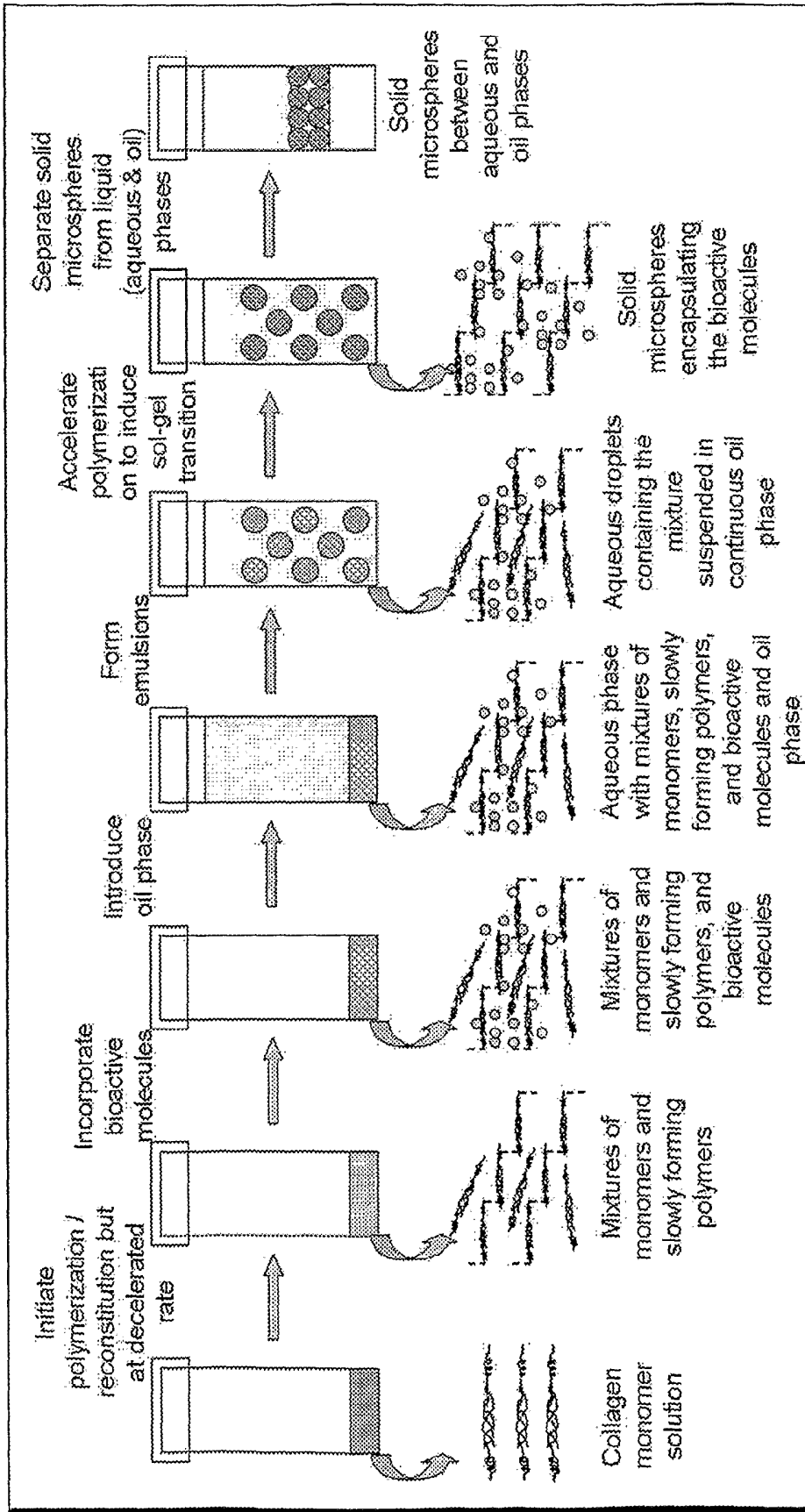


Figure 1

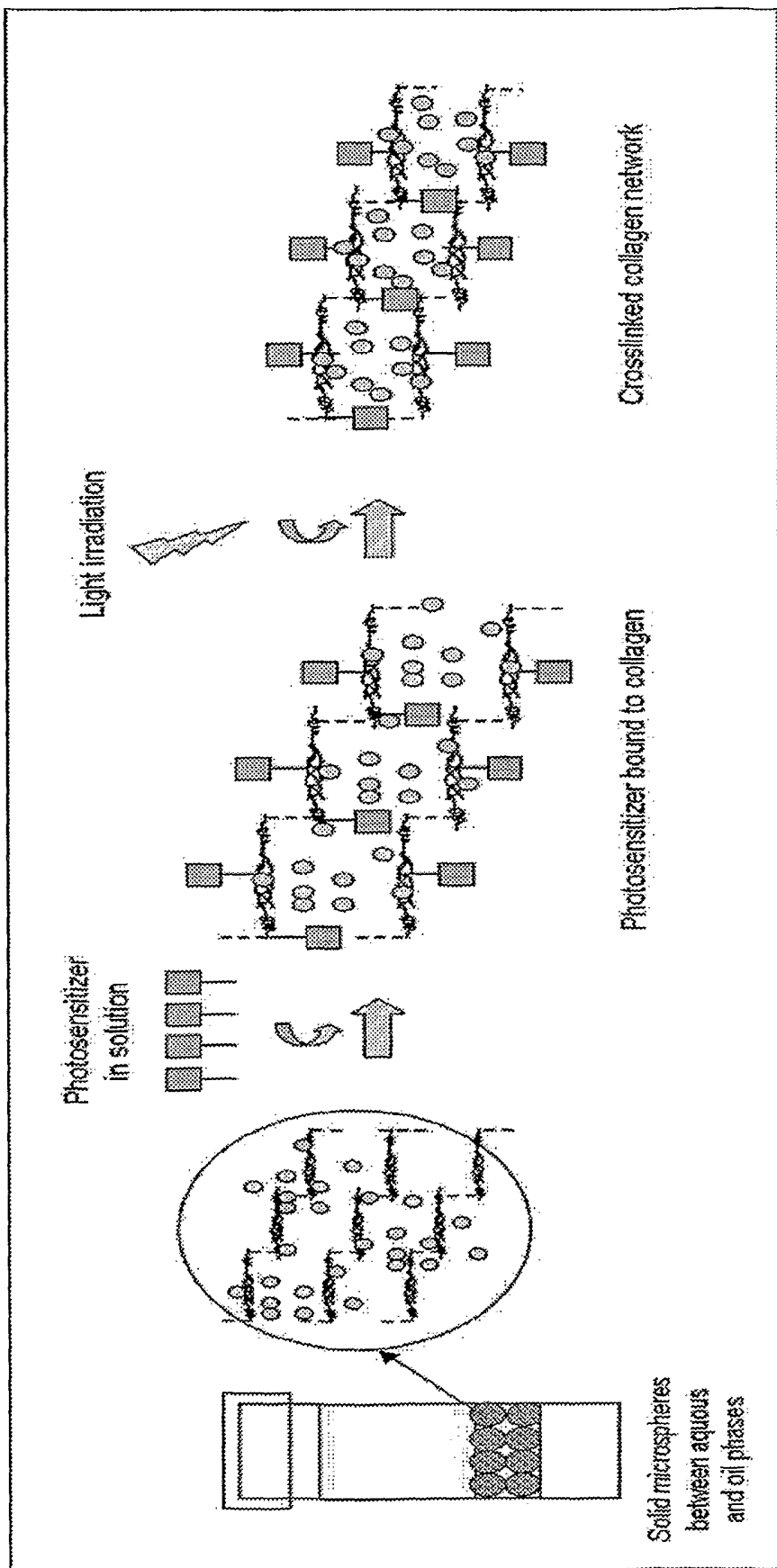


Figure 2

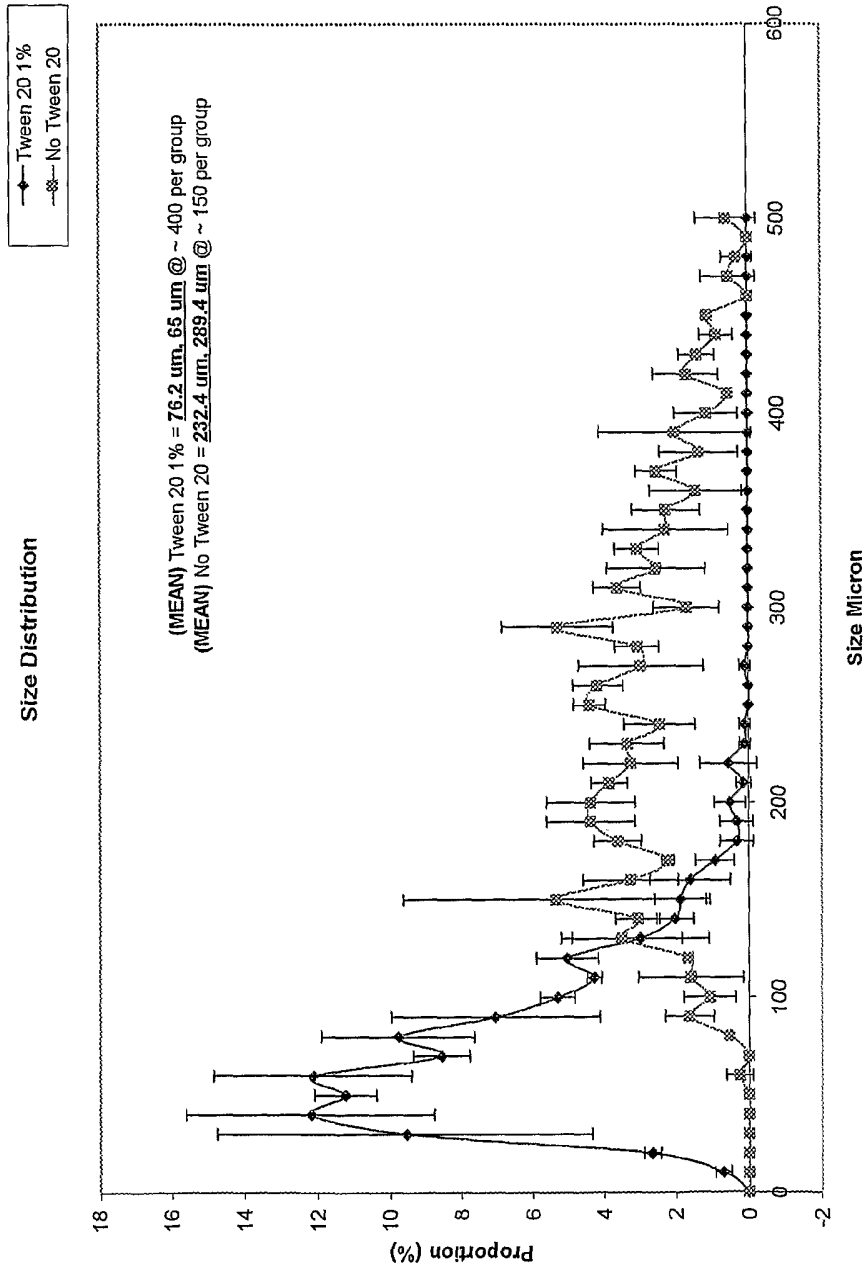


Figure 3

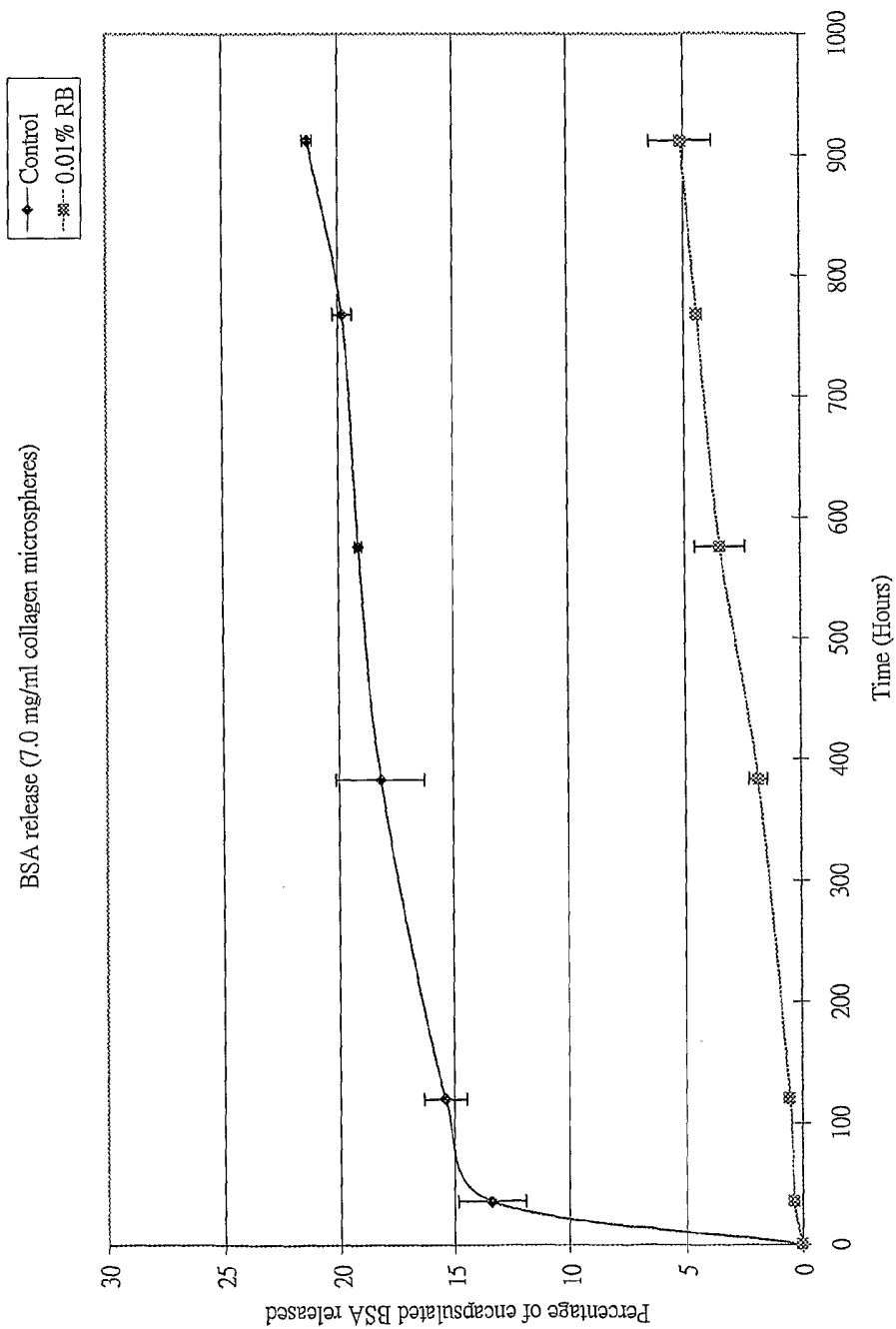


Figure 4

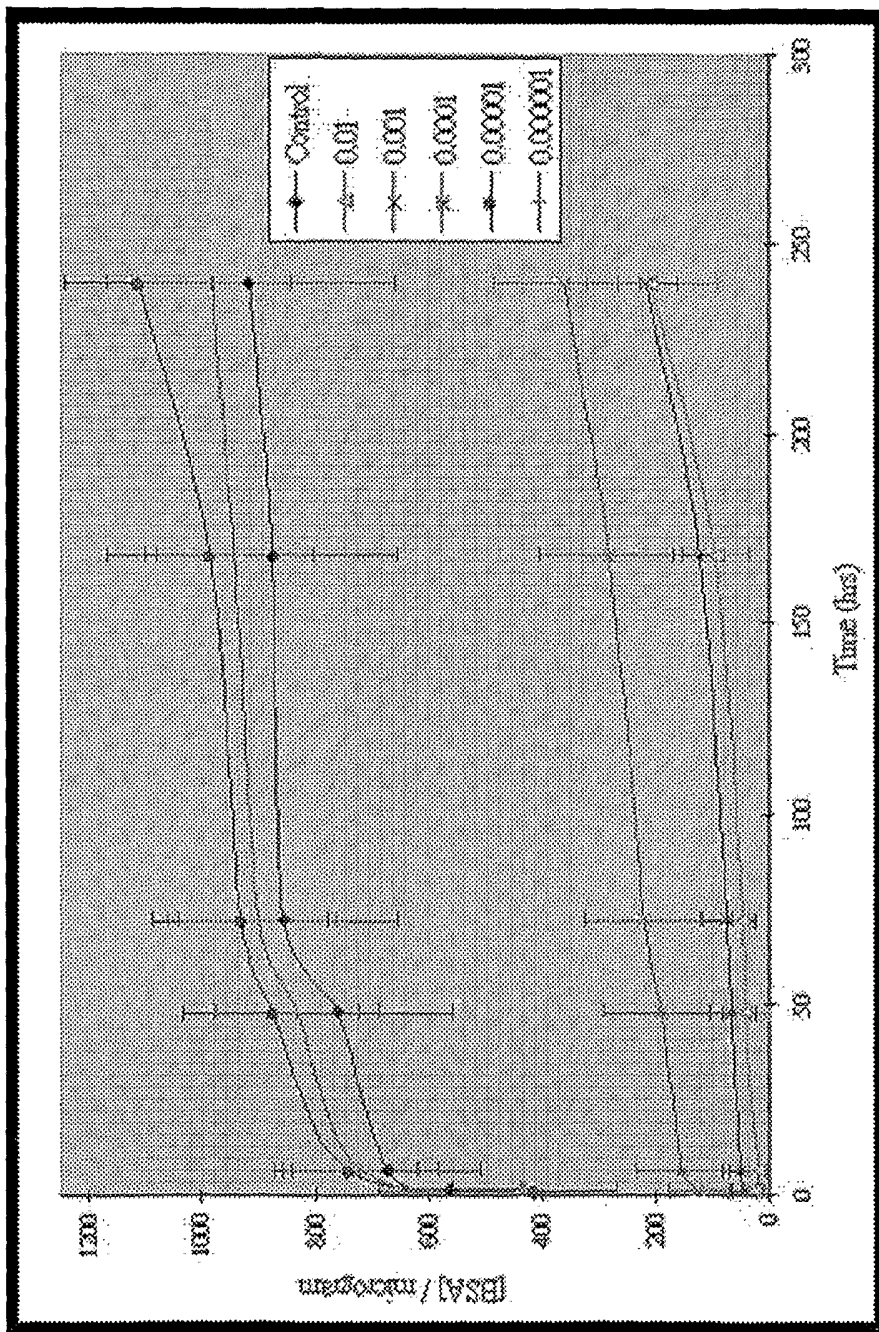


Figure 5

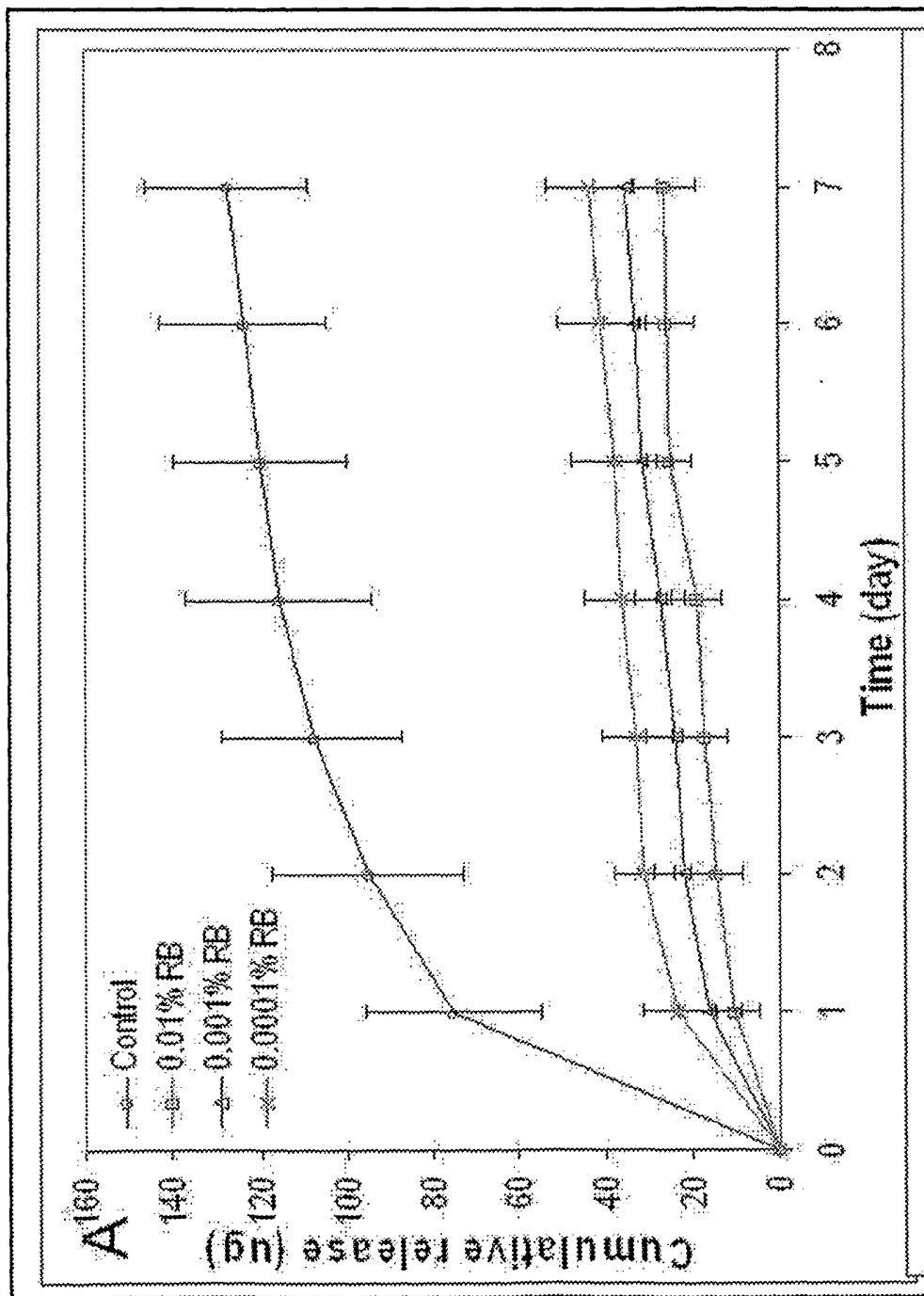


Figure 6

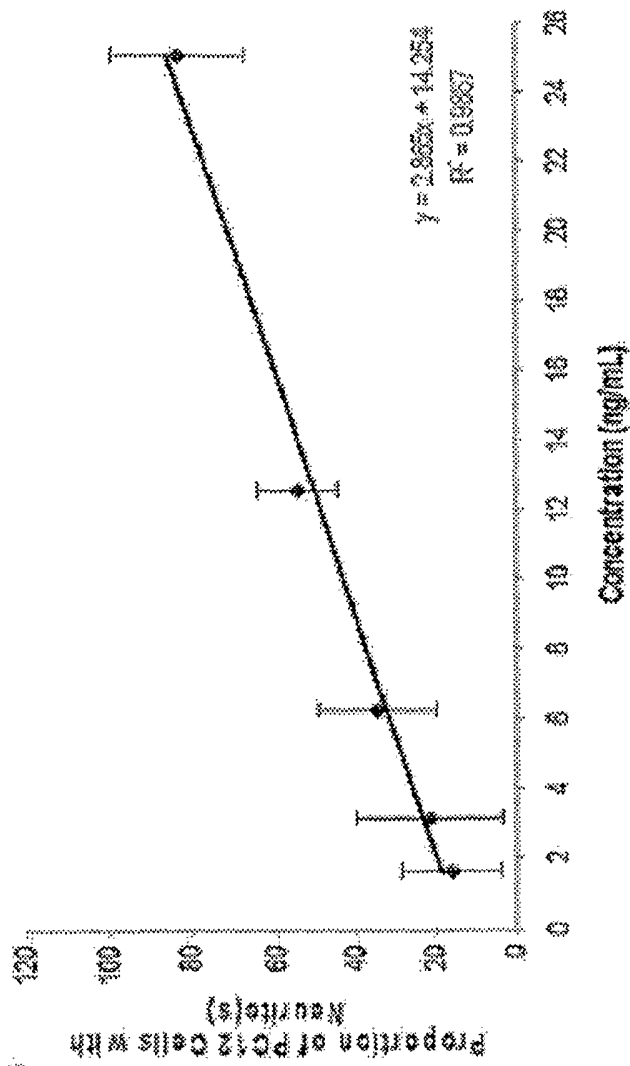


Figure 7

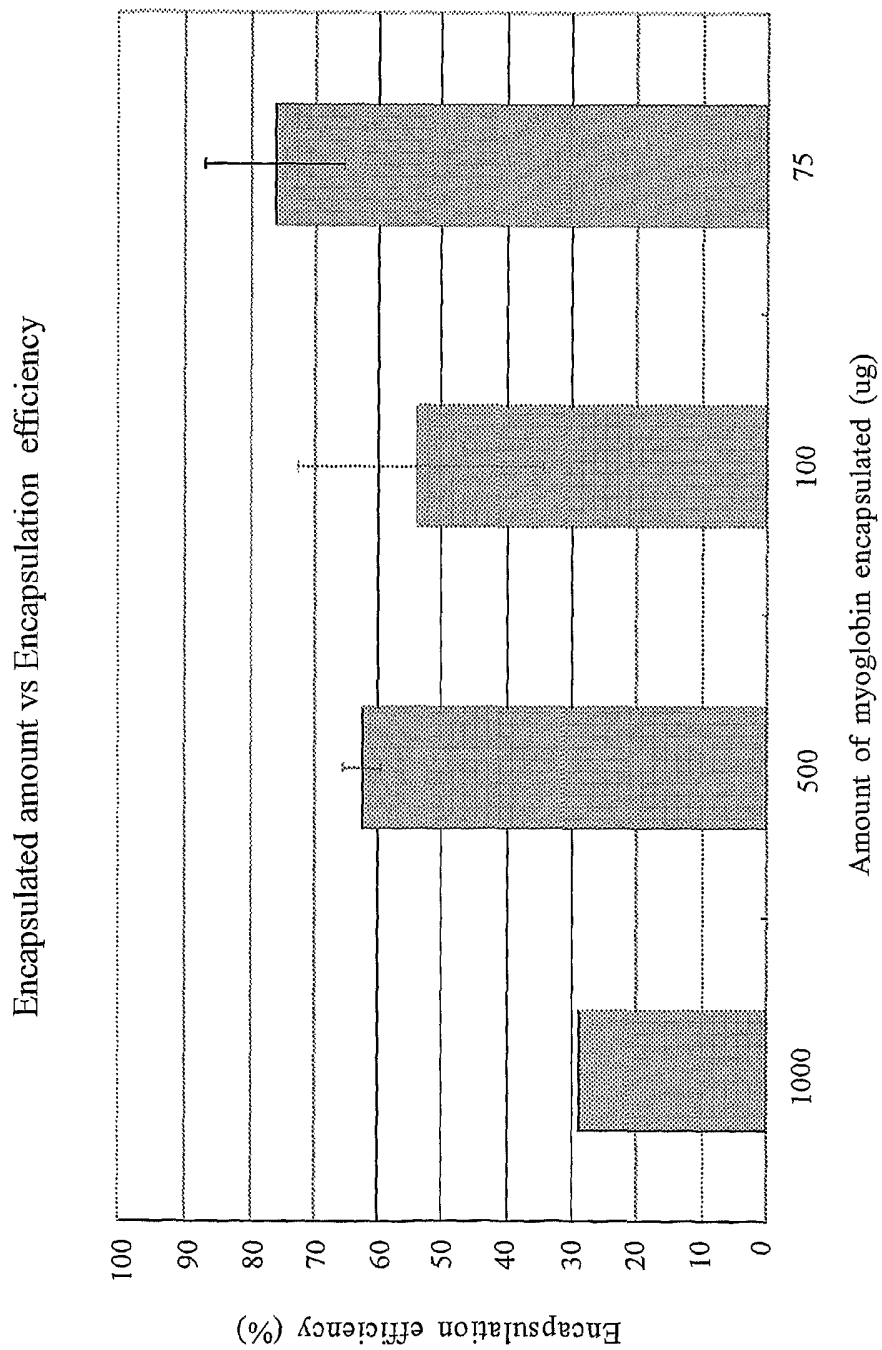


Figure 8

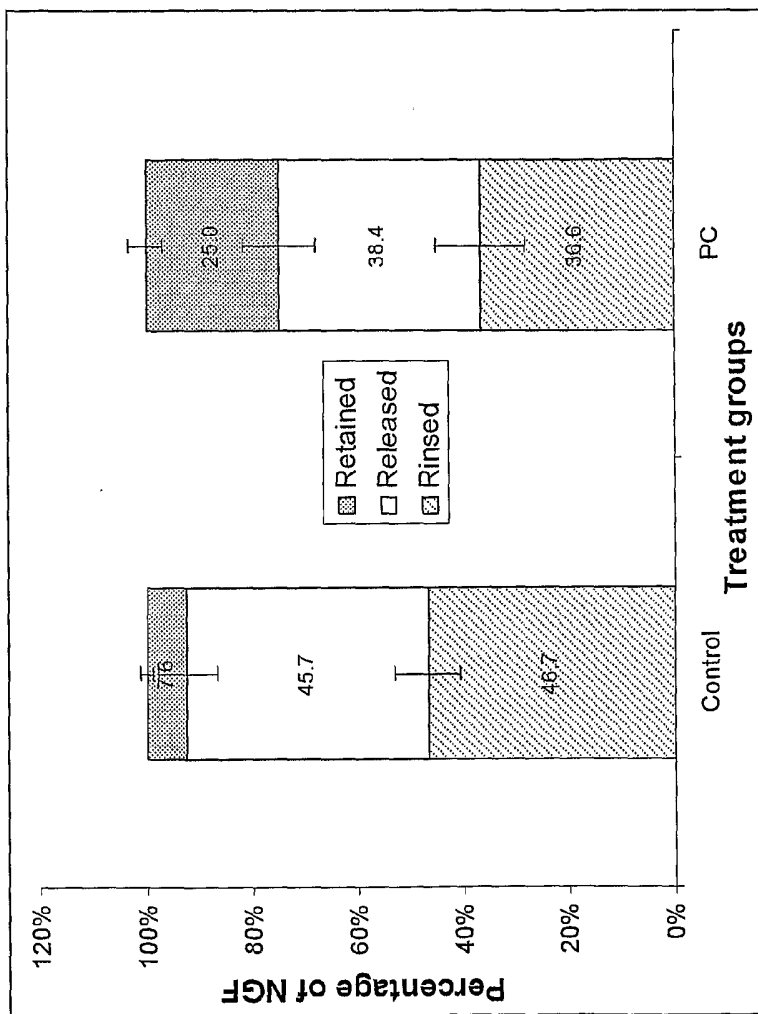


Figure 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2008/001277

A. CLASSIFICATION OF SUBJECT MATTER

See extra sheet

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: B01J 13/-; A61K; A61L

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)

WPI; EPODOC; PAJ; CNKI; CNPAT; ISI Web of Knowledge; extracellular; extra w cellular; collagen; microsphere?; micro w sphere?; microcapsule?; micro w capsule?; releas+; deliver+

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Shozo Miyazaki, et al. Fibrinogen Microspheres as Novel Drug Delivery Systems for Antitumor Drugs. Chem. Pharm. Bull. 1986, Vol. 34, No. 3, pages 1370-1375	1,4-15,17,18,24
Y		2,3,16,19-23
Y	WO2006029571A1(The University of Hong Kong) 23 March 2006(23.03.2006) page 11, line 20-23, page 12, line 20-24, page 13, line 12-29, example 1	2,3,16,19-23
PX	O.C.M. Chan, et al. Fabrication of nano-fibrous collagen microspheres for protein delivery and effects of photochemical crosslinking on release kinetics. Journal of Controlled Release 22 April 2008, Vol. 129, pages 135-143	1-24

Further documents are listed in the continuation of Box C.

See patent family annex.

<p>* Special categories of cited documents:</p> <p>“A” document defining the general state of the art which is not considered to be of particular relevance</p> <p>“E” earlier application or patent but published on or after the international filing date</p> <p>“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)</p> <p>“O” document referring to an oral disclosure, use, exhibition or other means</p> <p>“P” document published prior to the international filing date but later than the priority date claimed</p>	<p>“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</p> <p>“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</p> <p>“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</p> <p>“&” document member of the same patent family</p>
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Date of mailing of the international search report
23 OCT. 2008 (23.10.2008)

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2008/001277

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Takafumi Ishizaka, et al. Preparation of Egg Albumin Microcapsules and Microspheres. Journal of Pharmaceutical Sciences. April 1981, Vol. 70, No. 4, pages 358-363	1,4-15,17,18,24
Y		2,3,16,19-23
X	US3406119A(Keuffel & Esser Company) 15 October 1968(15.10.1968) examples 1-3	1,4-15,17,18,24
Y		2,3,16,19-23

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/CN2008/001277

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
US3406119A	15.10.1968	FR1470723A	24.02.1967
		GB1126856A	11.09.1968
		DE1542261A	26.03.1970
WO2006029571A1	23.03.2006	US2006099268A1	11.05.2006

Form PCT/ISA/210 (patent family annex) (April 2007)

CORRECTED SHEET

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2008/001277

B01J 13/14(2006.01)i

A61K 9/52(2006.01)i

A61K 9/50(2006.01)i