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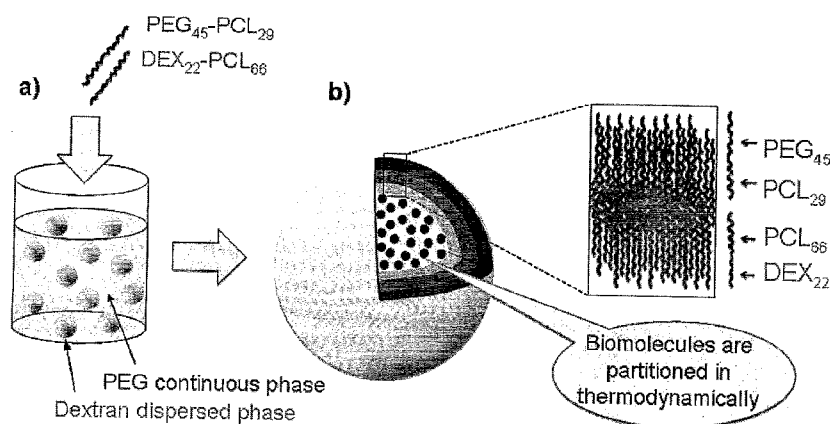


Fig. 1

(57) Abstract: This invention demonstrated a new polymer vesicle system structured with an asymmetric membrane which is formed from two amphiphilic di-block (A-B type) copolymers having different hydrophilic blocks or from a tri-block copolymer (A-B-C type) with two different hydrophilic blocks at each end. With this system bio-molecules or macromolecules can be nano- or submicron encapsulated by thermodynamic partition and be stabilized thermodynamically. This system is applicable in particulate and delivery of bio-therapeutics. What equally important in this invention is the method for forming polymer vesicles of asymmetric membrane: phase-guided self-assembly. This method involving hydrophilic two-phase system to guide the block copolymers above to align on the hydrophilic interface in designed orientation. This method will have great application in functionizing or bio-functionizing particulate surfaces.

Polymer Vesicles of Asymmetric Membrane

CROSS REFERENCE AND RELATED APPLICATIONS

5 This application claims priority of U.S. Serial No. 61/221,022 filed on June 26, 2009, the contents of which are incorporated by reference here into this application.

Throughout this application, reference is made to various publications. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this
10 application to more fully describe the state of the art to which this invention pertains.

Field of the invention

The present invention demonstrates a new particulate system called polymersomes of asymmetric bilayer membrane for which the vesicle membrane is formed from two types of
15 diblock copolymers, one forming the inner leaflet and the other forming the outer leaflet.

Background of the invention

Polymer vesicles, also called as polymersomes, possess the similar structure of lipid vesicles (or called liposomes), except that the membrane enclosing the aqueous core is
20 formed from amphiphilic block copolymers instead of lipids. Polymersomes have attracted great attention due to their superior mechanical stabilities over liposomes and the flexibility regarding chemical modification of the membrane forming copolymers. A number of different amphiphilic block copolymers, including poly(ethylene oxide)-*block*-poly(butadiene) (PEO-*b*-PBD), poly(ethylene oxide)-*block*-poly(ethylethylene) (PEO-*b*-PEE), poly(ethylene
25 oxide) -*block*-poly(lactic acid) (PEO-*b*-PLA), poly(ethylene oxide)-*block*-poly(caprolactone) (PEO-*b*-PCL), poly(ethylene oxide)-*block*-polystyrene (PEO-*b*-PS), have been used to form polymersomes. Differing from other polymeric particulates, on the other hand, polymersomes are unique in having the aqueous interior to which delicate biological therapeutics such as protein drugs, nucleotides, vaccines can be encapsulated with preserved native state. These
30 natures make polymersome to be a promising system for delivery of biological therapeutics.

Compared with the advances in biotechnology and rapid growth biological therapeutics market, the pace for development of biological delivery systems is far behind. The decades-long research efforts for sustained-release delivery and non-invasive delivery of
35 proteins have not yet resulted in a single product in these categories. For example, while the discovery of gene-silencing by siRNA has shown great potential to lead to revolutionary medicines,^[6-9] its application in disease treatment is blocked by lack of safe and effective system delivery systems to target cells and cell compartment.^[10, 11] The attempts to assemble

such systems encountered a series of difficulties. It is especially challenging to incorporate bio-molecules into particulate systems of sizes table o be taken up by target cells. The technical obstacles include limited loading efficiency, pre-targeting instability of the carrier systems, denaturing of bio-molecules during the formulation process, post-phagocytosis degradation, as well as formulation complexity.^[1-5]

The fact that many microorganisms can effectively deliver their nucleotide or peptide cargos into host cells suggests that the chemical mechanisms for inter- and intra- cellular trafficking of biological substances are available in the nature. Most microorganisms are composed of a hydrophilic interior isolated by in a stable and functional shell, allowing them to perform various bio-functions. The membrane of polymersomes possess a liquid crystalline structure alike many microorganisms as well the flexibility to be modified to simulate these functions. The polymersomes reported to date, however, lack a mechanism to encapsulate bio-macromolecules with sufficient capacity and stability, and a mechanism to facilitate endosomal escape after engulfing by cells. These limitations encouraged us to develop a new polymersome system to improve.

It is reported that the lipid compositions of the inner and outer leaflets of cell membranes are different from each other.^[12-14] In the plasma membranes of eukaryotic cells, phosphatidylcholine and sphingomyelin predominate in the outer leaflet while aminophospholipids are primarily in the cytosolic leaflet.^[12] The asymmetric structure of the cell membrane maintains the different composition and environment of intracellular and extracellular fluid. The asymmetric membrane of the biological cell membrane inspired us to develop polymersome of structure. With an asymmetric membrane, the chemical environment of a vesicle could be significantly different from that of the continuous phase, so that therapeutic molecules can be encapsulated in the vesicle by thermodynamic partition. Moreover, if the partition favors the vesicle interior, bio-molecules loaded in the vesicle may be stabilized thermodynamically due to reduced Gibbs free energy ($\Delta G = -RT\ln K_P$, where K_P is the partition coefficient of bio-molecules between the two aqueous phases).

To form a polymersome of asymmetric membrane, a method able to guide two different amphiphilic di-block copolymers to aform each leaflet of the vesicle bilayer is necessary. The method should also be able to guide tri-block copolymers to form a monolayer with defined orientation. Therefore, creating/utilizing a guiding system becomes a key factor for assembling vesicles of asymmetric membrane.

Summary of the invention

The present invention addresses the above-discussed technical issues by a

polymersome of asymmetric bilayer membrane and by a phase-guided self-assembly method. It combines the art of polymersome with the aqueous two-phase system to offer a polymersome with protein-friendly interior of dextran polymer. Upon preparation, two amphiphilic di-block copolymers having polysaccharide and polyethylene glycol (PEG) as the hydrophilic blocks, respectively, are added into an aqueous two-phase system consisting of polysaccharide dispersed phase and a PEG continuous phase. Because of the incompatibility of polysaccharide and PEG the phases, the block copolymers having polysaccharide block align at the interface of the aqueous two-phase system with its hydrophilic block facing the polysaccharide dispersed phase, while the one with PEG block align at the interface with its hydrophilic block facing the PEG continuous phase. If targeting or other functional molecules are conjugated to the distal end of the PEG block, the polymersome will be assembled in such a way surrounded by functional groups. Moreover, since most of water-soluble proteins and other biological agents favor polysaccharide phase more than PEG phase, these agents can easily be encapsulated are into the polysaccharide dispersed-phase by thermodynamic partition when being added in this system. Encapsulation by thermodynamically favored partition will greatly improve loading efficiency and stability of the agents to be packed. For protein encapsulation, the loading efficiency was reached 90% in experiment.

The polysaccharide core of the new polymersome offers numerical functions. In addition to the preferential partition of biological cargos, it may greatly improve mechanic stability of the polymersomes if the polysaccharide core is cross-linked through conjugated groups (glycidyl methacrylate conjugation for example). Furthermore, the conjugation linkage can be designed to be pH-sensitive so that the cross-linked polysaccharide core may degrade and dissociate inside the endosome of target cells. This nature is useful to design a delivery system facilitating its cargo's endosomal escape.

Finally, the materials used in this invention can be any biocompatible copolymer yielding self-assembled fully-biodegradable polymersomes necessary for human in vivo applications. The hydrophobic block of aliphatic polyester such as polylactic acid (PLA), polyglycolic acid (PGA), polylactic-co-glycolic acid (PLGA) and polyε-caprolactone (PCL), or polymers of similar structures are used. The bioactive agents feasible to load into polymersome of asymmetric bilayer membrane can be protein, DNA, siRNA, etc.

Brief description of the figures

Figure 1. Schematic description of the preparation procedure and structure of polymersomes of asymmetrical bilayer membrane. a) Preparation procedure by adding two block copolymers, DEX₂₂-PCL₆₆ and PEG₄₅-PCL₃₀, into a dextran/PEG aqueous two-phase system. b)

Polymersome structure consisting of a dextran core in which bio-macromolecules are packed by preferential partition and an asymmetric block copolymer bilayer shell with the dextran block facing the core and PEG block facing the PEG continuous phase. The two A-B types of diblock copolymer (DEX-PCL and PEG-PCL) may be replaced by a A-B-C type tri-block copolymer (DEX-PCL-PEG), and the dextran dispersed phase of the aqueous two-phase system may be replaced by solid particles such as polyplex.

Figure 2. Photo and microscopic images indicating formation of polymersomes of asymmetric bilayer. a) Photo images of FITC-dextran labeled dextran/PEG aqueous two-phase system added with no block copolymers (i), with PEG₄₅-PCL₃₀ alone (ii), with DEX₂₂-PCL₆₆ alone (iii), and with DEX₂₂-PCL₆₆ and PEG₄₅-PCL₃₀ together (iv). b) Microscopic images of Nile red labeled dextran/PEG aqueous two-phase system added with PEG₄₅-PCL₃₀ alone (ii), with DEX₂₂-PCL₆₆ alone (iii), and with DEX₂₂-PCL₆₆ and PEG₄₅-PCL₃₀ together (iv). c) Microscopic images of FITC-dextran labeled dextran/PEG aqueous two-phase system to which was added DEX₂₂-PCL₆₆ and PEG₄₅-PCL₃₀ together at a copolymer/dextran ratio of 0.25 (iv-1); 0.5 (iv-2); 1.0 (iv-3); and 3 (iv-4). Scale bars: 20 μm (b), 10 μm (c).

Figure 3. 2D- ¹H-NOESY-NMR spectrum of polymersomes in D₂O, recorded with a mixing time of 150 ms at 500 MHz. No special correlations between the DEX chain and the PEG chain was identified.

Figure 4. Laser confocal microscopic images of polymersomes of asymmetric bilayer membrane and lateral mobility of the membrane. a) Polymersomes labeled with Nile red and FITC-dextran scanned with a 559 nm laser beam for the single color image (i) and scanned with 559 nm and 488 nm beams in sequence for the combined image (ii). b) Experimental set-up for bleaching a region of a Nile red-labeled polymersome surface and imaging lateral diffusion. c) Confocal images of the Nile red-labeled vesicles taken at 0, 3, 14 and 28 seconds after bleaching treatment. d) Measured (the circles) and calculated (the line) relationship between relative fluorescent intensity (I_t/I_0) and post bleaching time. Scale bars: 10 μm

Figure 5. Microscopic images of polymersomes with and without core cross-linking. a) Fluorescent microscopic images of polymersomes without core cross-linking upon dilution of continuous phase by 0, 3 and 6 times. b) Microscopic images of polymersomes with cross-linked core. c) Microscopic (i) and fluorescent microscopic images (ii and iii) of the polymersomes in b) diluted by 10 times. d) Molecular structure of GMA-Dextran for forming cross-linked core. Scale bars: 10 μm .

Figure 6. a) Profile of cumulative EPO released from polymersomes of asymmetric bilayer

membrane *in vitro*. b) Profile of cumulative bioactivity of released EPO determined by UT-7 cell proliferation assay. Circles in each graph indicate the mean values of two measurements.

Figure 7. TEM images of dried polymersomes of asymmetric bilayer membrane with cross-linked core prepared by PEG-PCL and DEX-PCL in water (scale bar 200 nm).

Figure 8. Particle size distribution of polymersomes of asymmetric bilayer membrane formed from diblock copolymers of PEG-PCL and DEX-PCL by phase-guided assembly.

Detailed description of the invention

The present invention demonstrated a method (phase-guided self assembly) to form a unique composition of polymer vesicles (polymersomes of asymmetric bilayer membrane) with diameters ranging from sub-micrometers to micrometers. These and composition offer a convenient encapsulation of soluble proteins and other bio-molecules into submicron-sized particulate systems for drug therapy, immuno-therapy, gene therapy and other applications.

The amphiphilic molecules to form these polymersomes through the phase-guided assembly can be two different amphiphilic di-block copolymer (A-B type) or a tri-block copolymer with two different hydrophilic blocks at the two distal ends (A-B-C type). Some examples are hydrophilic polyethyleneoxide (PEG) conjugated to hydrophobic poly(caprolactone), hydrophilic dextran (DEX) linked to hydrophobic poly(caprolactone), or a poly(caprolactone) block conjugated with PEG and DEX at its two ends. These blocks were chosen for several favorable criteria such as their non-toxicity, biodegradability and known metabolism pathways *in vivo*. Due to the known safety and intensive hydrophilic property, PEG has been conjugated as the hydrophilic block for many block copolymers to form polymersomes. Dextran is widely used as blood substitution, drug carriers and aqueous phase material for protein purification because its sounded biocompatibility, biodegradability, hydrophilicity and high affinity to bio-macromolecules.^[20, 21] For its well demonstrated biocompatibility, biodegradability, PCL has already been widely used as drug delivery material.^[23, 24] An example for A-B-C type copolymer may be DEX-PCL-EPG. DEX may be replaced by other polysaccharide or oligo-sugars.

The polymersomes of the present invention are prepared by a unique phase-guided assembly method. For phase-guided assembly (also called self assembly), a hydrophilic two-phase system consisting of a dispersed phase and a continuous phase is needed as the "template". The continuous phase must be in liquid form while the dispersed phase can be in either liquid or solid form. Materials selected for forming the dispersed and continuous phases of the hydrophilic two-phase system should possess selective/biased affinity to the different

hydrophilic blocks of the block copolymers (defined as above), respectively. Polymeric vesicles of asymmetric membrane are formed by addition of the amphiphilic diblock copolymers (with different hydrophilic blocks) into the aqueous (or hydrophilic) two-phase system. An example of the hydrophilic two-phase system may be that consisting of an aqueous dextran solution as the dispersed phase and a PEG aqueous solution as the continuous phase. The DEX dispersed phase may be solidified by cross-linking treatment or be replaced by other aqueous solution or solid particles such as polyplex formed by cationic polymers and nucleotides. The intra-core cross-linking treatment may be achieved by covalent interaction or ionic (electrostatic) interaction between the polymer chains of the core (dispersed phase) material. The selective/biased affinity between the dispersed and continuous phases of the template two-phase system and the hydrophilic blocks of copolymer is based on various intermolecular interactions comprising electrostatic interaction, hydrogen bonding, or hydrodynamic repulsing (such as PEG to other macromolecules). As an example for electrostatic interaction, the sugar block of the polysaccharide (or oligo sugar)-PCL-EPG copolymer may be modified by charge-generating molecule, such as succinic anhydride.

In our examples, the selected dextran-PEG two-phase system works efficiently for protein purification due to the preferential partition favoring the dextran phase, as well as the protein stabilization effect of the dextran hydroxyls.^[15] In the preparation process, the copolymer with the dextran block aligned along the surfaces of the dextran droplets, under the guidance of phase separation, to form the inner leaflet of the bilayer; while the copolymer with the PEG block formed the outer layer, with the PEG chain facing the PEG continuous phase. Being enclosed by the asymmetric bilayer, the interior of the new polymersome possesses a different chemical environment from the continuous phase, and can therefore encapsulate biomolecules efficiently by thermodynamic partition. By conjugating targeted molecules to the distal end of the hydrophilic blocks of the copolymer forming the outer leaflet, polymersomes able to interact with specific cells may be assembled. Figure 1 schematically describes an example of this phase-guided assembly, as well as the structure of an asymmetric-bilayer polymersome.

The phase guidance strategy demonstrated in the present invention works well for preparing polymersomes of asymmetric bilayer membrane formed from two different diblock copolymers as well as polymersomes of asymmetric monolayer membrane formed from A-B-C triblock copolymers. In phase-guided assembly, hydrophilic-hydrophobic-hydrophilic triblock (A-B-C) copolymers align in an "A to A" and "C to C" style to form an asymmetric monolayer by designed phase selection. The portion and accuracy of phase-selection can be pre-determined by phase diagrams of selected systems or by partition test. There hundreds of reported phase diagrams for the so-called aqueous two-phase systems in the literature

including several textbooks ^[15]. While, as reported previously, the asymmetric monolayer could also be adjusted by the relative hydrodynamic size of the A block and the C block (the block having a smaller hydrodynamic size tends to align inwards and that of larger size aligns outwards), the efficiency of size-selection should be far below phase-selection. The phase-guided assembly demonstrated in this invention may offer a more effective and better defined assembly for block polymer orientation based on the well summarized phase diagrams of aqueous two-phase separation found in textbooks. For example, a dextran/PEG two-phase system in highly concentrated form, the amount of dextran dissolved in the PEG phase and vice versa is below 1.0 wt%. ^[15] Moreover, phase-guided assembly enables two well-selected amphiphilic diblock copolymers to form the inner and outer leaflets of a bilayer membrane, respectively, and with defined distribution.

Examples

Following examples are aimed to help technologists of related disciplines to better understand this invention. The examples should not be used to limit the applications and rights of this invention.

Example 1. Preparation of polymersomes of asymmetric bilayer membrane

To confirm the feasibility of the proposed phase-guided assembly process in forming polymersomes of asymmetric bilayer membrane, we added two diblock copolymers, such as PEG₄₅-PCL₃₀ and DEX₂₂-PCL₆₆ into a dextran/PEG aqueous two-phase system alone and jointly, followed by macroscopic and microscopic observation. The footnotes of molecular formula of each block copolymer indicate the number of repeating units of block. The aqueous two-phase system was prepared by mixing a dextran (70 KDa) solution (10% in concentration) and a PEG (8 KDa) solution (10% in concentration), with 0.1 wt% FITC labeled dextran, added to the dextran solution for convenient observation. In addition to PCL, polylactic acid (PLA), polyglycolic acid (PGA), polylactic-co-polyglycolic acid (PLGA), may be used as the hydrophobic block.

For direct observation, the prepared dextran/PEG two-phase system was then divided into four glass tubes: to each was added nothing, PEG₄₅-PCL₃₀ alone, DEX₂₂-PCL₆₆ alone, and both PEG₄₅-PCL₃₀ and DEX₂₂-PCL₆₆. The aqueous two-phase system with nothing added readily separated into two clear bulky phases after the stirring was ceased, with the fluorescently labeled dextran phase at the bottom and the PEG phase at the top (Fig. 2a-i). For the samples to which PEG₄₅-PCL₃₀ or DEX₂₂-PCL₆₆ were added alone, the system separated into two bulky phases (Fig. 2a-ii and 2a-iii). Moreover, the bulky phase of PEG became cloudy when was PEG₄₅-PCL₃₀ added (Fig. 2a-ii), and that of DEX became cloudy when DEX₂₂-PCL₆₆ was added (Fig. 2a-iii). Apparently, the two block copolymers partitioned

preferentially as aggregates into each of the phases which had the same content as their hydrophilic blocks. An interesting observation occurred when the two block copolymers, PEG₄₅-PCL₃₀ or DEX₂₂-PCL₆₆, were added together into the dextran/PEG aqueous two-phase system. The sample retained its "emulsion" form at least 60 minutes after stirring was stopped (Fig. 2a-iv). This result suggests that the two block copolymers thermodynamically prefer to accumulate at the interface between the dextran and PEG when added together.

To directly visualize the copolymers added in the dextran/PEG two phase system, we repeated the above experiment by replacing the FITC-dextran with a hydrophobic fluorescent dye, Nile red, and then observed under a microscope. For the aqueous two-phase system added with PEG₄₅-PCL₃₀ alone, the background (i.e. the PEG continuous phase) showed more intensive fluorescent emission, while the dextran dispersed phase were fluorescently less active (Fig. 2b-ii). This result agrees with the FITC-dextran labeled experiment that PEG₄₅-PCL₃₀ was distributed in the PEG phase. Similarly, when DEX₂₂-PCL₆₆ was added into the dextran/PEG two-phase system alone, the dextran dispersed phase became more fluorescently intensive (Fig. 2b-iii). For the sample with the two copolymers, PEG₄₅-PCL₃₀ and DEX₂₂-PCL₆₆, together, Nile red was distributed at the surface of the dispersed phase (Fig. 2b-iv). The Nile red labeled experiment provides further evidence for the accumulation of PEG₄₅-PCL₃₀ and DEX₂₂-PCL₆₆ at the interface between the dextran dispersed phase and the PEG continuous phase when they were added together.

More evidence for this particular distribution of the block copolymers at the interface of dextran/PEG two phase system is given by the relationship between the particle sizes of dextran dispersed phase and amount of PEG₄₅-PCL₃₀ and DEX₂₂-PCL₆₆ added together to the two-phase system. The dextran dispersed phase was again labeled with FITC-dextran. As shown in Figure 2c-iv, as the copolymer-to-dextran ratio (wt/wt) is increased from 0.25 to 0.5 and to 1.0, the average diameter of the dispersed phase decreases from 9.2 μm to 5.3 μm to 2.2 μm , respectively. Further increases in the copolymer-to-dextran ratio to 3.0 resulted in invisibility of the dextran dispersed phase under optical microscopes. Clearly, the two copolymers, when added together into dextran/PEG two-phase system, favored the dextran-PEG interface thermodynamically and created more interfacial membrane (reflected by the reduced particle sizes of the dextran dispersed phase, i.e. increased specific surface area).

The above three experiments (Fig. 2), taken together, suggest that the two copolymers, PEG₄₅-PCL₃₀ and DEX₂₂-PCL₆₆, formed an asymmetric bilayer structure at the interface of the dextran/PEG aqueous two-phase system, with the dextran and PEG blocks in contact with their respective similar phases.

Example 2. Cross-interaction between DEX- and PEG- blocks after forming polymersomes

2D-¹H-NOESY-NMR spectroscopy was reported as a reference method to indicate/suggest whether has a PEG block and a sugar group of polymersomes formed from A-B-C tri-block molecules. According to the literature reports,^[16-18] 2D-¹H-NOESY-NMR spectrometry is capable of identifying the interaction between individual molecules packed in a physical distance comparable to that of adjacent groups within a molecule by cross peaks. Figure 3 shows chemical shift peaks of the proton signals of methane in PEG units at 3.57 ppm and that of dextran units at 3.60-3.65 ppm, 3.53-3.35 ppm, respectively.^[19] No cross-interaction peak between dextran and PEG was observed. The absence of the hydrogen bonding interactions between the PEG and dextran blocks may suggest that PEG block and dextran block are not mixed together in the bilayer membrane.^[16] While NMR spectrometry may not be capable to identify small portion mixing of the DEX- and PEG-blocks into each other's phase, the result (Fig. 3) is convincing that majority of the DEX- and PEG- blocks were not mixed. Therefore, the structure of the polymersomes seems only possible in an asymmetric bilayer membrane, as illustrated in Figure 1. This result supports our previous conclusion that the PEG block and the dextran block of the two amphiphilic diblock copolymers were aligned facing the two sides of the dextran/PEG interface, respectively.

Example 3. Lateral diffusion of vesicle membrane of polymersomes of asymmetric bilayer

In the present invention, lateral mobility of the asymmetric copolymer bilayer was examined using time-based laser confocal imaging. The hydrophobic and hydrophilic fluorescent dyes (Nile red and FITC-dextran) were used to label the hydrophobic shell of vesicular membrane and the hydrophilic core, respectively. Figure 4a-i and 4a-ii show the laser confocal image of Nile red-labeled surface and the combined image of the surface and core labeled by each of the dye, respectively. Clearly, Nile red and FITC-dextran are distributed in the surface and the core of the dispersed dextran phase, respectively. This result agrees with our previous conclusion that the two diblock copolymers formed a bilayer around dispersed dextran. To examine lateral mobility of the surface membrane, a laser confocal beam of a laser confocal microscope (LSCM) was parked over a portion of a polymersome to induce local photo-bleaching of the Nile red causing an opening in the ring of its fluorescent image (Fig. 4b, 4c-i). Then, confocal images across the bleached area were taken at determined intervals after the bleaching treatment. The opened ring gradually closed over 28 seconds (Fig. 4c-ii, 4c-iii, and 4c-iv), suggesting that unbleached Nile red molecules diffused into the bleached region from the surroundings. Figure 4d shows the time course of fluorescence recovery in the bleached area. Although the diffusion rate of the dye molecule is not necessarily the same as that of the block copolymers, the rapid diffusion of hydrophobic

Nile red can only occur in a mobile and continuous polymer bilayer.

Since the vertical extension of the bleached region is significantly longer than its horizontal extension, and the confocal plane is thin (Fig. 4b), the lateral diffusion rate of Nile red may be estimated using a one-dimensional model (along the confocal plane) of Fick's law. The diffusion coefficient of Nile red along the surface, D , may be calculated by incorporating the measured values of fluorescent intensity of the bleached region and time into the earlier-state solution of Fick's second equation

$$I_t / I_0 = 4 \left(Dt / \pi L^2 \right)^{1/2} \quad (1)$$

where L is the length of the opening of the fluorescent circle in cm, t is time after bleaching treatment in seconds, I_t and I_0 are the measured fluorescent intensities above the background at time t and before bleaching, respectively. Based on the measured data, D was calculated, using the least squares method, to be $2.67 \times 10^{-9} \text{ cm}^2 \cdot \text{s}^{-1}$. Inserting the values of D and L into the equation resulted in a curve consistent with the measured data (Fig. 4d). This value is comparable to that of phospholipid bilayers ($10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$).^[20]

Example 4. Modification of the core structure of polymersome of asymmetric bilayer membrane

The core of the polymersome may be endowed with a capability to respond to intracellular pathways. If the core is solidified by a pH sensitive cross-linking mechanisms, it could retain the shape in neutral environments but dissociate and burst at low pH endosomes by acid-driving cleavage of the intra-core linkages. The microscopic and fluorescent images shown in Figure 5 demonstrate the core property, based on which desired functions can be incorporated. The polymersomes whose core matrices were not cross-linked were enlarged and ruptured when the PEG continuous phase was removed or diluted (Fig. 5a-i, 5a-ii and 5a-iii). When the core matrix was formed of methacrylate-grafted dextran and therefore cross-linked by radical polymerization using ammonium peroxydisulfate (APS) and N,N,N',N'-tetramethylethylenediamine (TEMED) (0.2 wt% and 0.4 wt% in concentration, respectively) as an initiation system, then it can endure the abrupt change of the osmotic pressure, and dilution of the PEG continuous phase no longer caused enlargement and rupturing of the polymersomes (Fig. 5b, 5c-i, 5c-ii, and 5c-iii). Cross-linked core matrices can effectively hold the bilayer membrane. Breaking the matrix cross-linking will lead to immediate rupture of the particulate. This key characteristic is especially useful for delivering biomolecules into the cytoplasm of target cells through phagocytosis (endosomal escaping).

Example 5. Encapsulation of biomolecules in polymersome of asymmetric bilayer and release of biomolecules from the polymersomes

Further provided are encapsulation and controlling the release of fragile biomolecules from the polymersomes of asymmetric bilayer membrane. For example, erythropoietin (EPO) was added in the phase-guided assembly process, followed by assays of protein content, release profile and bioactivity (Fig. 6a). The encapsulation efficiency of the protein was 89% measured by the micro-BCA method, which is dramatically higher than the reported 5% for polymersomes with symmetric bilayers.^[1] In addition, Figure 6 shows that the release kinetic profile of EPO from the polymersomes (with a cross-linked core) is identical to the cumulative bioactivity profile by UT-7 cell proliferation assay (Fig. 6b), suggesting that protein activity was well preserved in this particulate system. In addition, since proteins loaded in the interior of polymersome are stabilized by dextran, they can endure 40 °C for 6 h and no protein aggregation increase observed by HPLC method.

Example 6. Preparation of nano-sized polymersome with asymmetric bilayer polymersome

The procedure for preparing nanometer-sized polymersomes of asymmetric bilayer membrane was extended from the method in Example 1 by increasing the copolymer/dextran ratio. In brief, 1 mL diblock copolymer solutions, 5 mg · mL⁻¹ in concentration, was prepared by incubating for 12-24 h at 60 °C with or without magnetic stirring. Then PEG/ GMA-dextran (400 mg / 100 mg) and protein were dissolved in 4 mL KCl (0.22 M) solution flushed for 10 min with nitrogen and subsequently transferred into the above diblock copolymer solution followed by vortexing for 1 min. The system was then incubated for 1 h at 40-50 °C to cross-link the interior dextran of the polymersome by the addition of ammonium peroxydisulfate (180 ul, 50 mg/mL) and N,N,N,N tetramethylethylenediamine (100 ul, 20% V/V, adjusted to pH 7 with 4 M HCl). The polymersomes with cross-linked lumen were characterized by TEM and DLS (See Figure 7 and Figure 8)

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15

WHAT IS CLAIMED IS

1. A method to prepare polymer vesicles of asymmetric membrane, comprising
 - a) preparing a hydrophilic two-phase system consisting of a hydrophilic dispersed phase
5 and a hydrophilic continuous phase;
 - b) adding block copolymers having two different hydrophilic blocks into the hydrophilic two-phase system prepared in step a); and
 - c) stirring, shaking, sonicating, or mixing (with other method) the sample prepared in step b).
- 10 2. The method of claim 1, wherein the block copolymers having two different hydrophilic blocks may be two amphiphilic di-block (A-B type) copolymers having different hydrophilic blocks or one (or more) amphiphilic tri-block (A-B-C type) copolymer(s) having two different hydrophilic blocks conjugated at the two end of the hydrophobic block.
3. The method of claim 1, wherein the dispersed phase of the hydrophilic two-phase system
15 may be in liquid form or in solid form.
4. The method of claim 1, wherein the two different hydrophilic blocks possess selective affinity to the dispersed and the continuous phases of the hydrophilic two-phase system, respectively.
5. The method of claim 3, wherein the liquid form dispersed phase may be solidified by
20 intra-core cross-linking through covalent and ionic (electrostatic) interaction.
6. The method of claim 1, wherein the hydrophilic two-phase system consists of polysaccharide dispersed phase and a PEG (or PEO) continuous phase.
7. The method of claim 5, wherein the cross-linking treatment may involve usage of polysaccharide conjugated with a molecule possessing a cross-linkable bond.
- 25 8. The method of claim 6, wherein the polysaccharide dispersed phase is formed from an aqueous solution of dextran, starch, inulin, carboxylic methyl cellulose, or other cellulose derivatives.
9. The method of claim 1, wherein the hydrophilic two-phase system consists of hydrophilic solid dispersed phase and a PEG (or PEO) continuous phase.
- 30 10. The method of claim 9, wherein the hydrophilic solid dispersed phase may be polyplex, particles formed of nucleotides or cationic polymers.
11. The method of claim 1, wherein bio-molecules may be added at any step of the preparation process to be encapsulated into the polymer vesicles.
12. The method of claim 11, wherein the bio-molecules are selected from proteins, peptides,
35 DNA and RNA.
13. A polymeric vesicle system prepared by the method of claim 1, comprising a hydrophilic core which is formed from the hydrophilic dispersed phase of the hydrophilic two-phase system and asymmetric membrane which is formed from the block copolymers.

14. The polymeric vesicle system of claim 13, wherein the hydrophilic core may be in liquid form or solid form.

15. The polymeric vesicle system of claim 14, wherein the hydrophilic core in liquid form is polysaccharide solution.

5 16. The polymeric vesicle system of claim 14, wherein the hydrophilic core in solid form is cross-linked polysaccharide or polyplex particles.

17. The polymeric vesicle system of claim 13, wherein the block copolymers may be two amphiphilic di-block (A-B type) copolymers having different hydrophilic blocks or one (or more) amphiphilic tri-block (A-B-C type) copolymer(s) having two different hydrophilic blocks

10 conjugated at the two end of the hydrophobic block.

18. The polymeric vesicle system of claim 17, wherein the A-B type amphiphilic di-block copolymers are a polysaccharide block conjugated with a hydrophobic polymer block and a PEG block conjugated with a hydrophobic polymer block.

15 19. The polymeric vesicle system of claim 18, wherein the hydrophobic polymer blocks are biodegradable polymers.

20. The polymeric vesicle system of claim 19, wherein the biodegradable polymers are selected from polylactic acid (PLA), polyglycolic acid (PGA), polylactic-co-polyglycolic acid (PLGA), and polyε-caprolactone (PCL).

21. The polymer vesicle system of claim 13, wherein bio-molecules may be encapsulated.

20 22. The polymer vesicle system of claim 21, wherein the bio-molecules are selected from proteins, peptides, DNA and RNA.

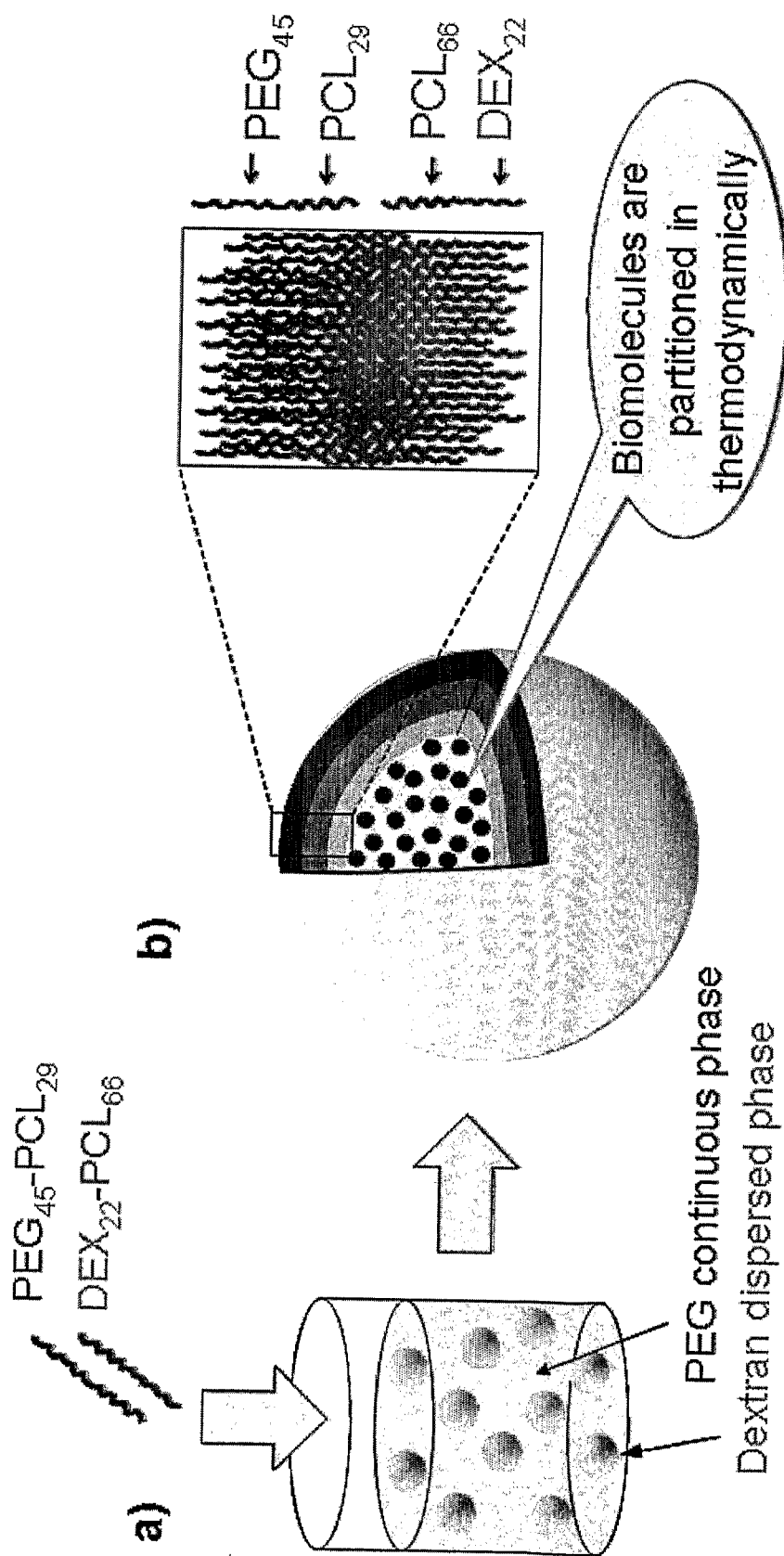


Fig. 1

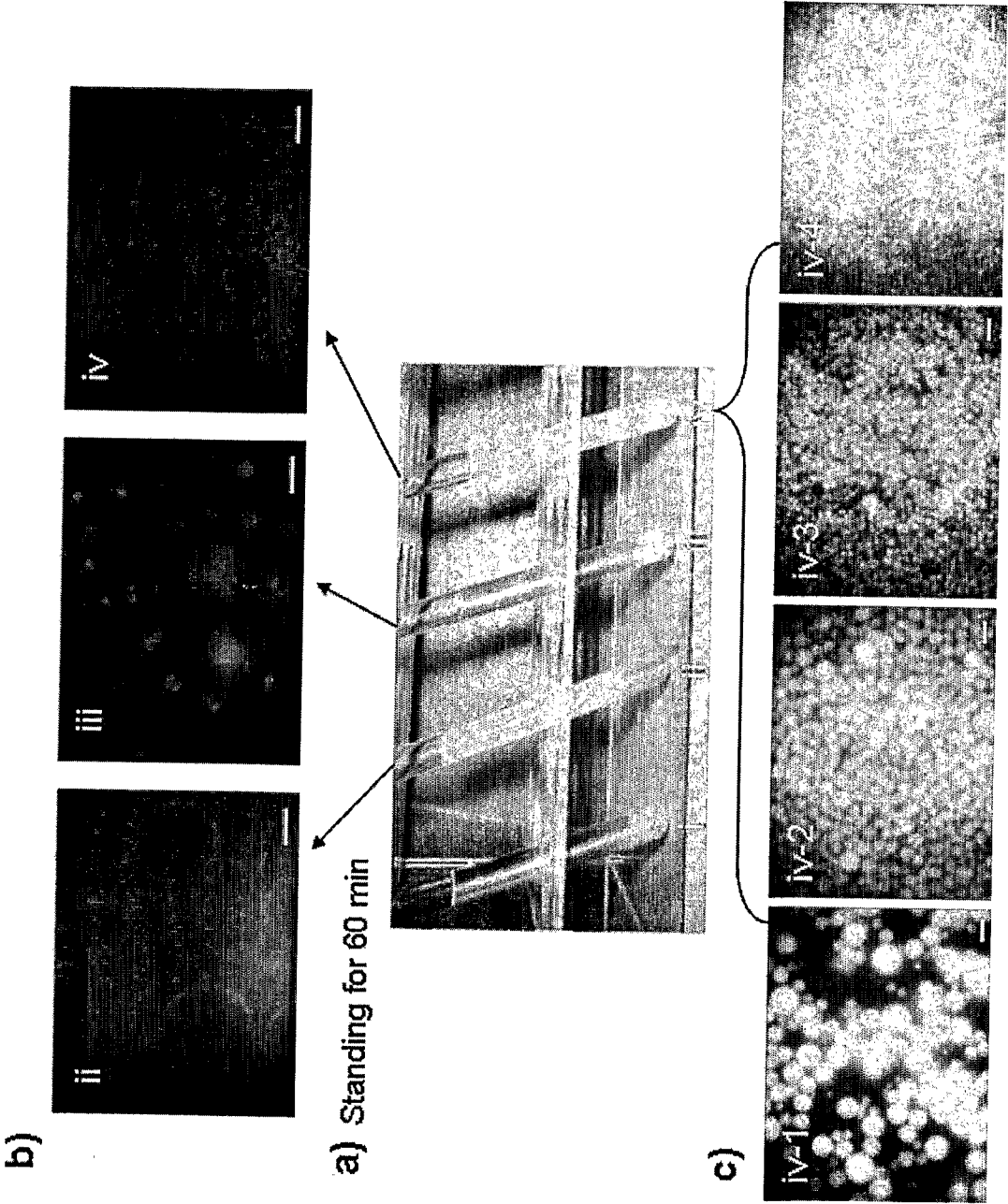


Fig. 2

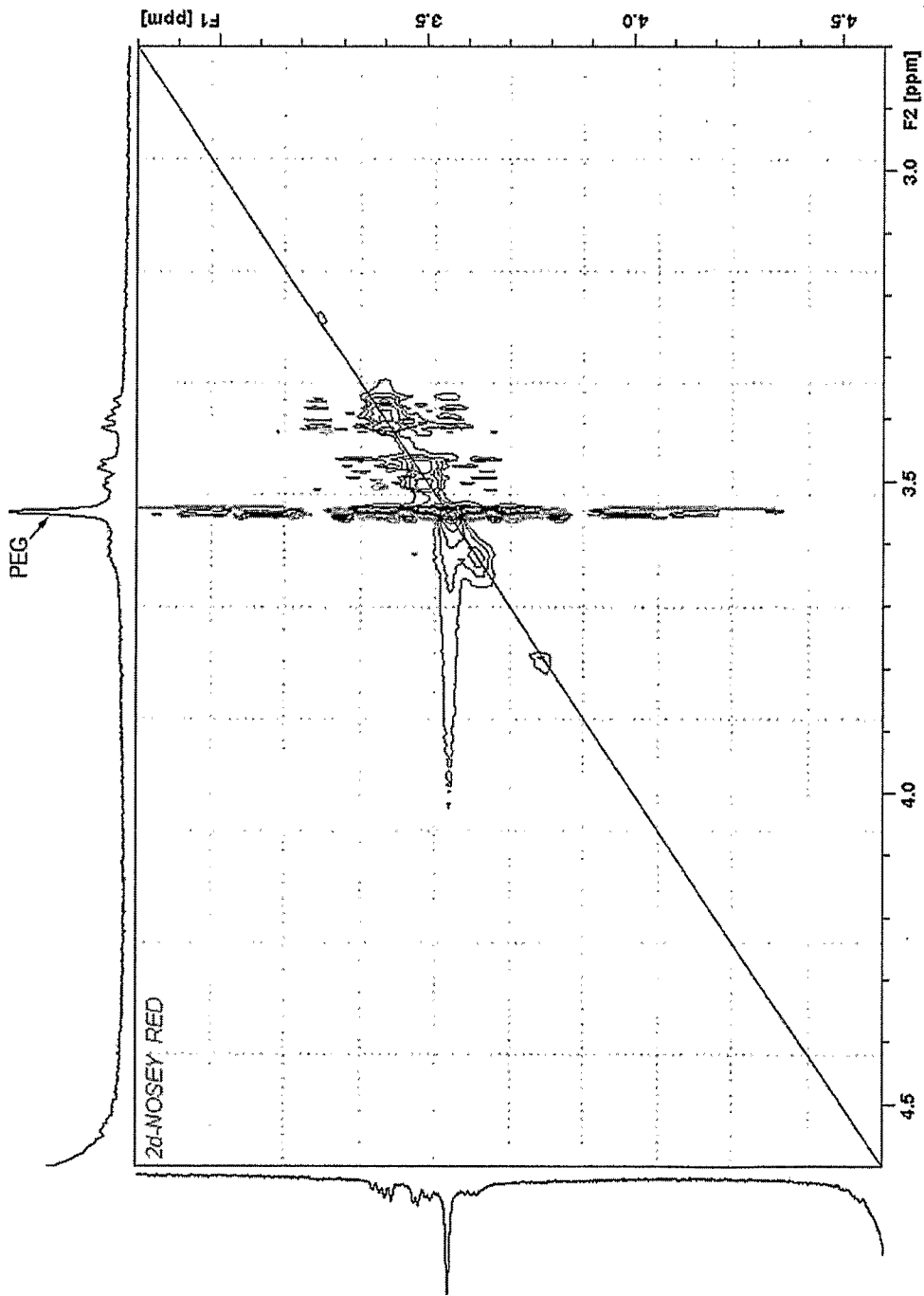


Fig. 3

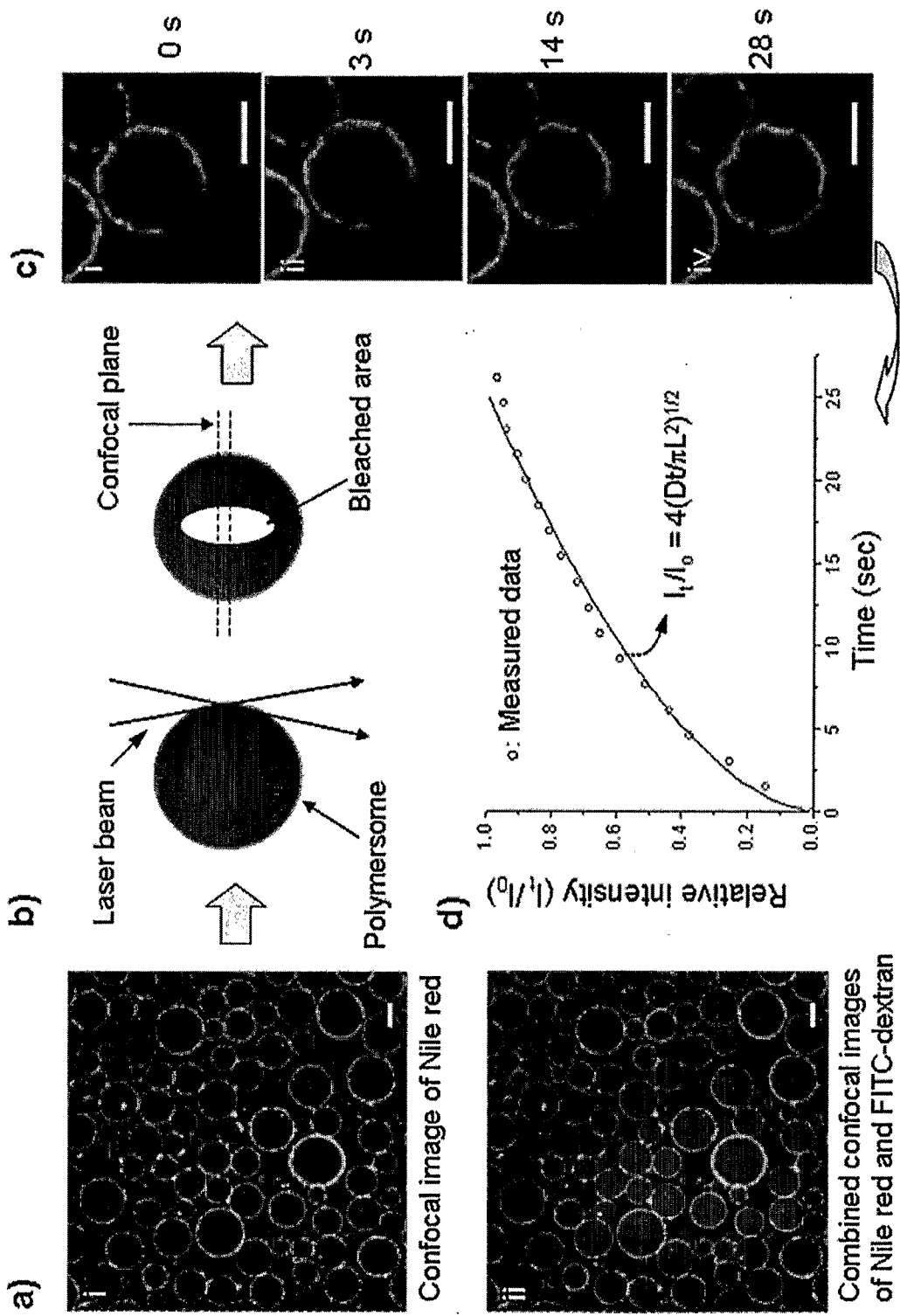


Fig. 4

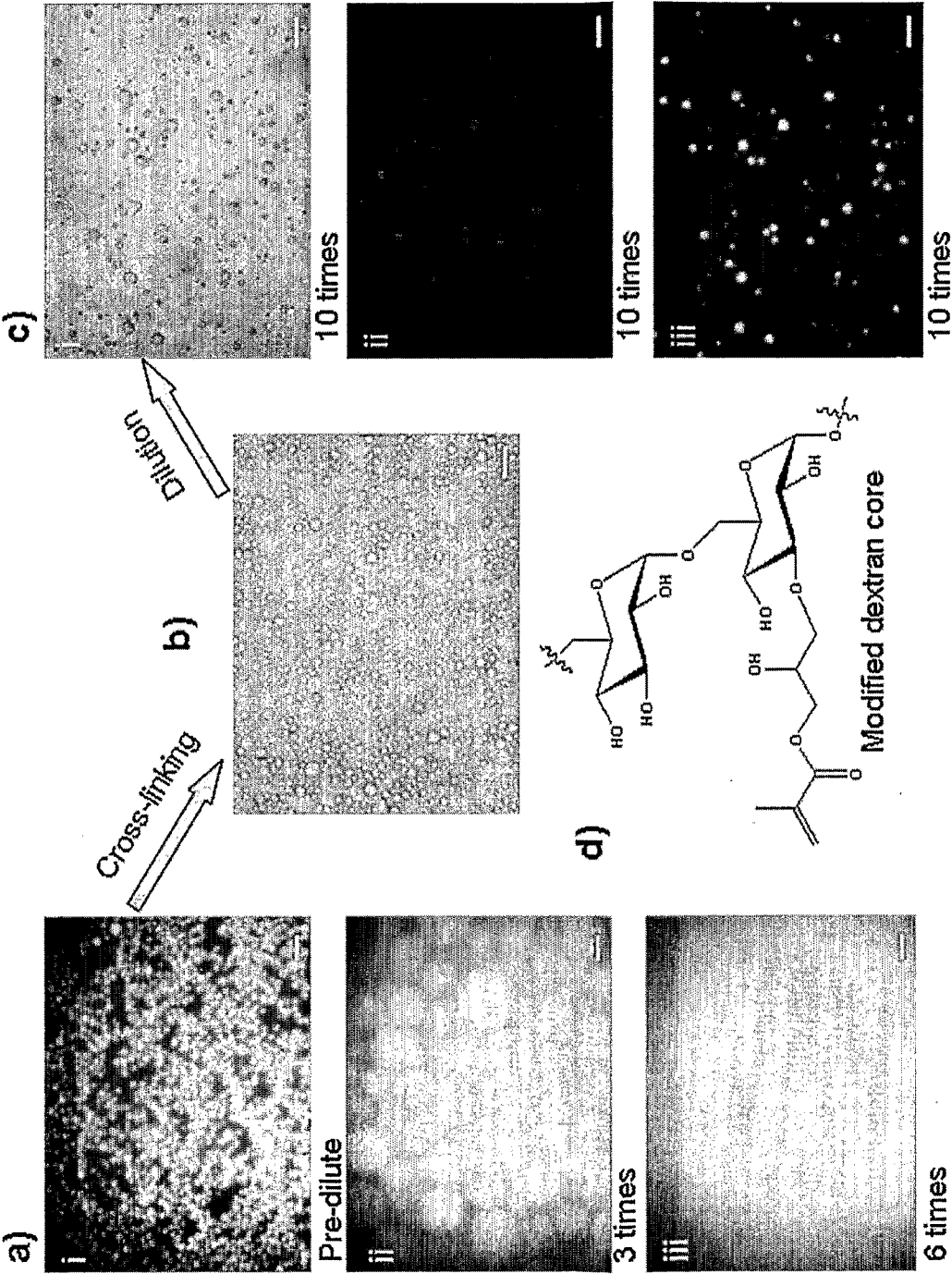


Fig. 5

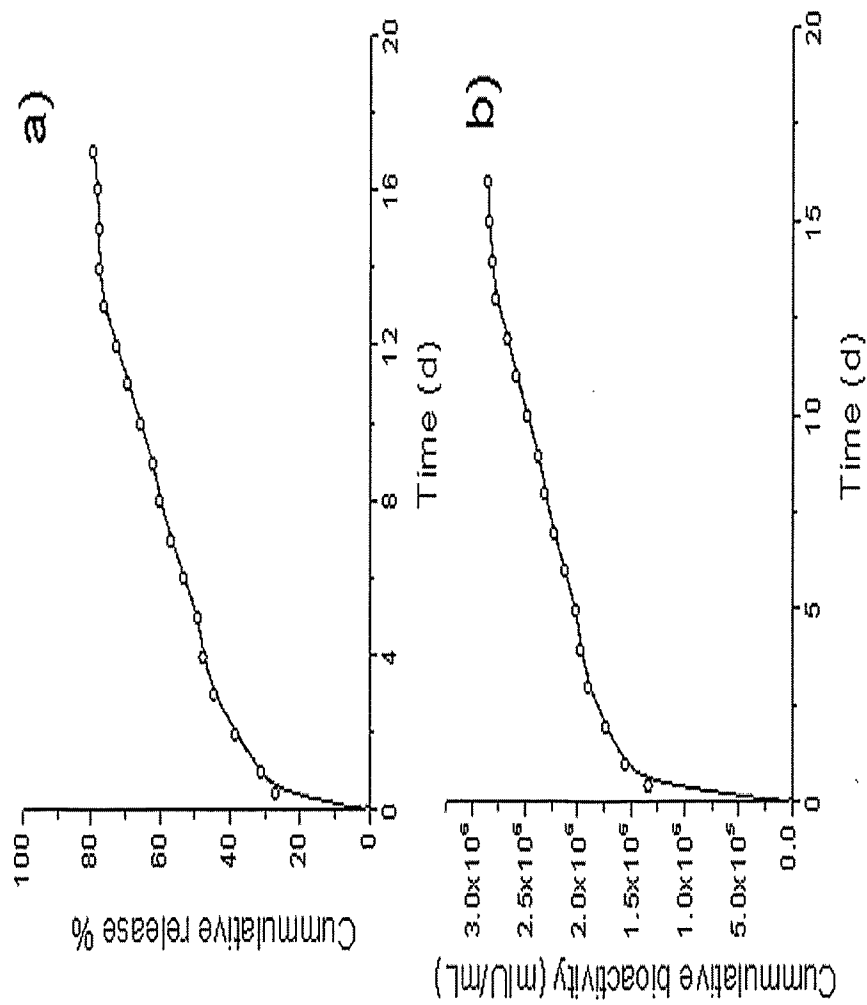


Fig. 6

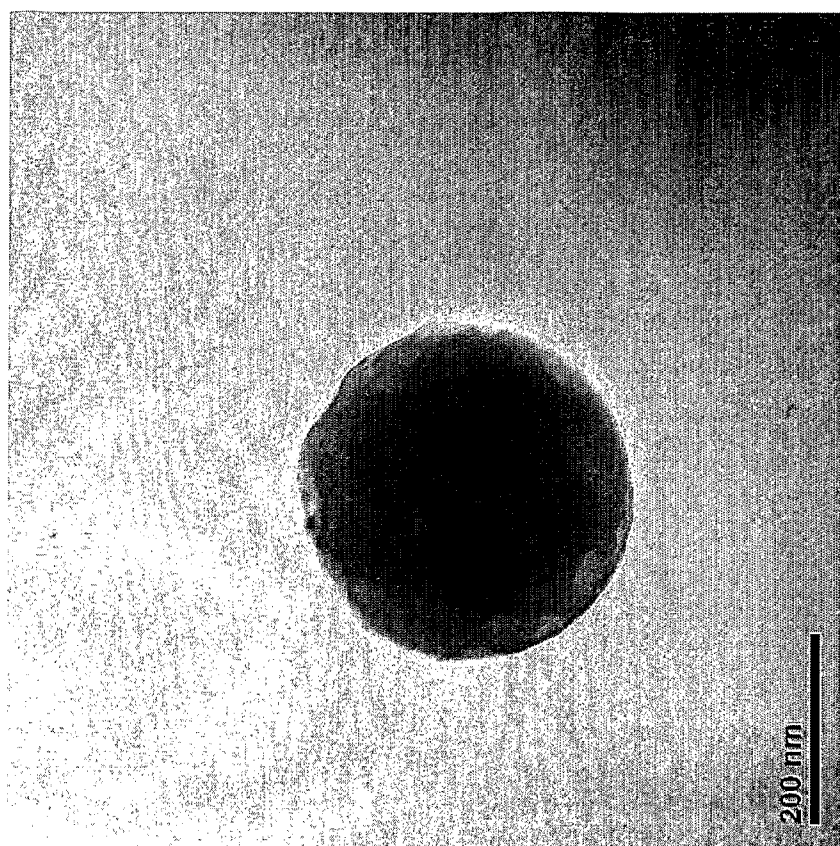


Fig. 7

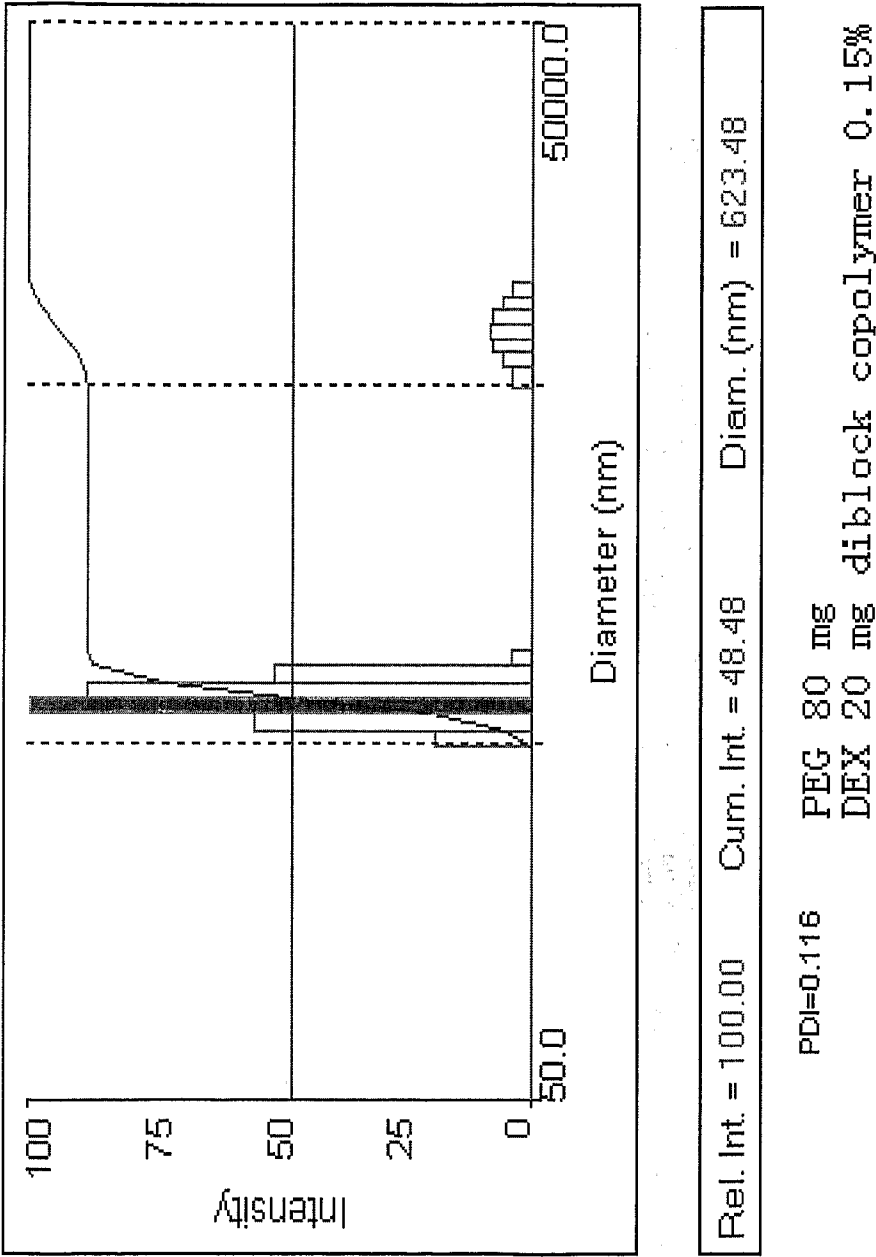


Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2010/000968

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: A61K9/-, 31/-, 47/-; C08F;C08J3/-;

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)

CNPAT,CNKI,WPI,EPDOC: VESICLE? OR POLYSOME? HYDROPHILIC OR AMPHIPHILIC (BLOCK??? OR SEGMENT???) W ??POLYMER? POLYSACCHARIDE? OR DEXTRAN? OR STARCH?? OR INULIN? OR CELLULOSE? PEG OR PEO OR (POLYETHYLENE W GLYCOL?) OR (POLYETHYLENE W OXIDE?) HOLLOW W PARTICLE?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US2008305149A1(AMJI M ET AL.) 11 Dec.2008(11.12.2008) See paragraphs [0020], [0033], [0040], [0043], [0045], [0047]	13,14,17,21-22
X	US2004010060A1(HARVARD COLLEGE ET AL.) 15 Jan. 2004(15.01.2004) See paragraphs [0023]-[0028], [0056]-[0069], [0192]-[0217]	13,14,17,21-22
E	CN101791516A(SUZHOU UNIVERSITY) 04 Aug. 2010(04.08.2010) See paragraphs [0006]-[0013]	13,14,17,21-22

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:	“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
“A” document defining the general state of the art which is not considered to be of particular relevance	“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
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“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)	“&”document member of the same patent family
“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	

Date of the actual completion of the international search 27 Sep.2010(27.09.2010)	Date of mailing of the international search report 28 Oct. 2010 (28.10.2010)
Name and mailing address of the ISA/CN The State Intellectual Property Office, the P.R.China 6 Xitucheng Rd., Jimen Bridge, Haidian District, Beijing, China 100088 Facsimile No. 86-10-62019451	Authorized officer HE,Fang Telephone No. (86-10)62084472

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2010/000968

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US2008260833A1(BIOCURE INC ET AL.) 23 Oct.2008(23.10.2008) See paragraphs [0036]-[0042]	1-22
A	US2008181939A1(HENDON SEMICONDUCTORS PTY LT) 31 Jul.2008(31.07.2008) See the whole document	1-22
A	US2005003016A1(AHMED F ET AL.)06 Jan. 2005(06.01.2005) See the whole document	1-22

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2010/000968

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US2005003016A1	06.01.2005	NONE	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2010/000968

CLASSIFICATION OF SUBJECT MATTER(According to International Patent Classification (IPC) or to both national classification and IPC)

A61K9/51(2006.01)i

A61K31/715(2006.01)i

A61K31/74(2006.01)i

A61K47/34(2006.01)i

C08J3/07(2006.01)i