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(54) Title: COMPOSITIONS OF LESS IMMUNOGENIC AND LONG-CIRCULATING PROTEIN-LIPID COMPLEXES

(57) Abstract: Provided are lipidic particles comprising phopatidylcholine, phosphatidylinositol and cholesterol. Also provided
are compositions comprising the lipidic particles and having associated therewith therapeutic agents such as peptides, polypeptides
or proteins. In these compositions, the therapeutic agents have reduced immunogenicity and/or longer circulating time. These com-
positions can be used for therapeutic administration of the peptides, polypeptides and/or proteins.

COMPOSITIONS OF LESS IMMUNOGENIC AND LONG-CIRCULATING PROTEIN-LIPID COMPLEXES

This application claims priority to U.S. Provisional application no. 60/787,411 filed
on March 30, 2006; U.S. Provisional application on 60/787,586 filed on March 30, 2006;
U.S. provisional application no. 60/865,062 filed on November 9, 2006 and U.S.
Provisional application no. 60/870,177, filed on December 15, 2006, the disclosures of
which is incorporated herein by reference.

BACKGROUND OF THE INVENTION

In the treatment of diseased conditions, therapeutic interventions are often
undertaken which involve administration of foreign molecules having therapeutically
beneficial effects. However, such administrations can often result in unwanted side effects
resulting from activation of the body's immune response. Formation of antibodies
following administration of therapeutics poses a serious clinical challenge. The antibodies
can abrogate activity and/or alter pharmaco-kinetics of the therapeutic molecules.

This is particularly relevant when administering strong antigenic molecules such as
peptides, polypeptides or proteins. Many such polypeptides are routinely used as therapeutic
molecules. For example, Factor VIII (FVIII) is an essential cofactor in the intrinsic
coagulation pathway. Any deficiency or dysfunction of FVIII results in a bleeding disorder,
characterized as hemophilia A. Replacement therapy with recombinant FVIII (rFVIII) or
plasma-derived FVIII (pdFVIII) is the common therapy for controlling bleeding episodes.
FVIII is a multidomain glycoprotein comprising of six domains (A1-A2-B-A3-C1-C2).
Prior to secretion into plasma, FVIII is subjected to proteolytic cleavage, leading to the
generation of a heterodimer with molecular weights ranging from ~ 170 to ~300 KDa. The
presence of the multiple proteolytic sites at the B domain level is responsible for the high
heterogeneity of the FVIII preparations. In spite of being FVIII's largest domain (908
amino acids residues or ~ 40% of the total number of amino acids residues), the B domain
lacks any essential function for the cofactor coagulation activity. Deletion of the B domain
leads to a less heterogenic, genetically engineered rFVIII that corresponds to the shortest

form of pdFVIII (e.g. ~170KDa). B domain deleted rFVIII (BDDrFVIII) is characterized by a higher specific activity than rFVIII and can also be used for treatment of hemophilia.

Another therapeutic molecule is Factor VIIa (FVIIa). This is a trypsin-like serine protease which plays an important role in activating the extrinsic coagulation cascade.

5   FVIIa is a poorly catalytic form of factor VII after the activating cleavage between Arg152 and Ile153. Upon injury, circulating FVIIa becomes an efficient catalyst when forming a complex with tissue factor (TF), its allosteric regulator that is found on the outside of blood vessel. FVIIa-TF complex induces generation of small amounts of thrombin which further triggers blood clotting. Factor VIIa has been approved by the Food and Drug

10   Administration in the United States for uncontrollable bleeding in hemophilia A and B patients who have developed inhibitory antibodies against replacement coagulation factors, factor VIII and factor IX. Intravenous administration of recombinant human Factor FVIIa (rHu-FVIIa) has been introduced because of fewer side effects than other alternative treatment strategies and to circumvent difficulty in preparing plasma-derived FVIIa.

15   However, the short circulation half-life of FVIIa requiring repeated bolus injections to achieve desired efficacy can be problematic.

Additionally, many other proteins are used as therapeutics. These include erythropoietin, VEG-F, other blood coagulation proteins, hormones (such as insulin and growth hormone) and the like. Strategies that can inhibit processing by immune system and

20   also prolong circulation time (reduce frequency of administration) would improve efficacy of proteins. Therefore there is a need in the area of therapeutics to develop formulations that make the proteins less immunogenic, without significantly affecting the circulating time or the efficacy.

25   SUMMARY OF THE INVENTION

The present invention provides compositions comprising therapeutic agents such that the immunogenicity of the agents is reduced and their circulating time is increased. The compositions comprise lipidic particles (also referred to herein as lipidic structures) comprising phopatidylcholine, phosphatidylinositol and cholesterol. Therapeutic agents

30   such as peptides, polypeptides and/or proteins can be associated with the lipidic particles to form delivery compositions.

In these compositions, the therapeutic agent displays reduced immunogenicity and longer circulating time.

In various embodiments, lipidic particles having associated therewith proteins such as Factor VIII, B domain deleted Factor VIII, Factor VII, lysozyme and Erythropoietin are disclosed.

In the description, the therapeutic agent associated with the lipidic particles comprising PI is sometimes referred to as therapeutic agent-PI.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of biophysical and biochemical characterization of Laurdan study of PC containing liposomes alone as a liquid or gel or PI containing lipidic particles with associated rFVIII.

Figure 2. Biophysical and Biochemical characterization of rFVIII-PI (a): Transmission Electron Micrograph (TEM) of rFVIII-PI; (b); normalized fluorescence emission spectra of free rFVIII and rFVIII-PI (1:10,000); (c): a list of capture monoclonal antibodies utilized in this study that target specific epitopes in rFVIII molecules; (d): the binding of monoclonal antibodies to rFVIII-PI at various lipid concentrations. Control is protein free liposomes. (e): far-UV CD spectra of rFVIII in the presence (1:2,500) and in the absence of PI acquired at 20 °C; and (f): percent change in ellipticity of rFVIII as a function of temperature in the presence and in the absence of PI.

Figure 3. Effect of phophatidylinositol on the Immunogenicity of rFVIII. (a, c) show the mean of total antibody titers (horizontal bars) and individual (open circles) antibody titers were determined following s.c. and i.v. administrations, respectively. (b, d) show the mean of inhibitory titers (horizontal bars) and individual (open circles) inhibitory titers were determined following s.c. and i.v. administrations, respectively.

Figure 4. Influence of phosphatidylinositol on pharmacokinetics of rFVIII. The mean plasma concentration of rFVIII clotting activity after i.v. administration of free rFVIII and rFVIII-PI.

Figure 5. Influence of phosphatidylinositol on pharmacokinetics of BDDrFVIII. The mean plasma concentration of BDDrFVIII clotting activity after i.v. administration of free BDDrFVIII and BDDrFVIII-PI.

Figure 6. Acryamide quenching for free lysozyme and lysozyme associated with PI containing lipidic particles.

DESCRIPTION OF THE INVENTION

The present invention provides methods and compositions for less immunogenic and long circulating lipidic formulations for delivering a therapeutic agent. The formulations comprise a therapeutic agent associated with lipidic structures comprising phosphatidyl choline (PC), and phosphatidyl inositol (PI) and cholesterol. The therapeutic agent may be a peptide (generally 50 amino acids or less) a polypeptide(generally 100 amino acids or less) or proteins (larger than 100 amino acids).

Although not intending to be bound by any particular theory, it is considered that the lower imunigenicity and/or the longer circulation time is at least in part due to the lipidic particles having a unique structure. As seen under high magnification, the lipidic particles of the present invention do not appear to have donut like structures typical of liposomal lamellarity. Substantial number of the lipidic particles displayed disc like structures (see Example 2) which is attributable to reduced water volume thereby providing reduced contrast in the electron micrographs. Therefore the morphology appears different from that of liposomes, possibly due to altered lipid structure and organization, and reduced internal water volume. To further investigate the lipid structure and organization we carried out fluorescence studies using Laurdan as probe. The probe partitions into the interfacial region and the emission is sensitive to the presence and dynamics of water molecules and lamellar structures of liposomes. The fluorescence emission spectra were acquired for Laurdan labeled lipid particles of the present invention and also for liposomes, the latter serving as control. For liposomes that undergo transition from gel to liquid crystalline phase a red shift in the emission maxima, from 440 nm to 490 nm is observed (Figure. 1). Based on the composition one would expect an emission spectrum corresponding to liquid crystalline phase. However, laurdan labeled lipidic particles of the present invention showed a spectrum that is neither gel like nor liquid crystalline like. Thus, the data indicates that lamellar organization in this particle is different from that of liposomes - possibly due to the water concentration and dynamics being altered in this particle. Centrifugation studies carried out in discontinous dextran gradient indicated the particle floated more readily than liposomes. Thus, the lipidic structures of the present invention appear to have altered lipid organization and dynamics, internal water volume, water concentration and/or dynamics near the head group compared to typical liposomes. In addition, the particle may be lighter than the lamellar liposomes.

The association efficiency of the proteins in the lipidic particles as well as the reduction in the immunogenicity of proteins associated with the lipidic particles comprising

PI was greater than for similar compositions in which PI was replaced with PS, PA or PG. Since PI, PS, PA and PG are all anionic phospholipids, the advantage obtained by using PI was surprising. Further because one of the proteins tested, FVIII is known to bind more avidly to PS than to PI, it was surprising that the association efficiency of FVIII for PI

5   containing lipidic structures was higher than that for PS containing liposomes.

The present invention also provides a method for preparing the lipidic structures. The lipidic structures can be prepared by thin lipid film hydration using the appropriate molar ratios of PC, PI and cholesterol in a suitable buffer. The lipids are dissolved in chloroform and the solvent is dried. The resulting multilamellar vesicles (MLVs) are

10 extruded through the desired size filters (sizing device) under high pressure to obtain lipidic structures of the present invention. It is generally preferred that the size of the lipidic particles should be less than 140 nm (as calculated from micrographs and dynamic light scattering measurements) so that the particles are not filtered out in the Reticulo Endothelial System (RES) so as to become available for the immune system reaction. Thus it is

15 preferred to have at least 50% of the particles to be less than 140 nm More preferably, the particles should be less than 120 nm and still more preferably between 40 and 100 nm. In various embodiments, 50, 60, 70, 80, ad 90% of the particles are less than 140nm and more preferably between 40 and 100 nm.

To effect association of the protein with the lipidic structures, the protein in a

20 suitable buffer is added to the lipidic structures. The free protein is then separated from the the lipidic structures by routine centrifugation methods such as density gradient centrifugations. In various embodiments, the association efficiency of the protein with the lipidic particles is at least 30, 40, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95%. It desired, the lipidic particles with the associated therapeutic agent can be lyophilized for future use.

25 In one embodiment, the lipidic structures of the present invention prior to association with the protein can be lyophilized and stored. When needed, the lipidic structures can be reconstituted and then used for combination with protein to effect association of the protein with the lipidic structures prior to use.

The present invention can be used for association of therapeutic agents such as

30 proteins, polypeptides or peptides with the lipidic structures. The protein and peptides with wide biochemical properties can be loaded in the particles. The proteins may be neutral or charged (negatively or positively). Such proteins include proteins involved in the blood coagulation cascade including Factor VIII (FVIII), Factor VII (FVII), Factor IX (FIX), Factor V (FV), and von Willebrand Factor (vWF), von Heldebrant Factor, tissue

plasminogen activator, insulin, growth hormone, erythropoietin alpha, VEG-F, Thrombopoietin, lysozyme and the like.

The ratio of PC to PI to cholesterol can be between 30:70:1 to 70:30:33. Thus the ratio of PC to PI can vary between 30:70 to 70:30. In one embodiment, it is between
5   40:60: to 60:40 and in another embodiment 45:55 to 55:45. In another embodiment, it is 50:50. The cholesterol (as a percentage of PC and PI together) is between 1 and 33% as structures formed at higher cholesterol ratio than 33% lack stability. In one embodiment, the cholesterol is 5-15%.

The association of the protein with the lipidc structures can be such that the molar
10   ratio between the protein to lipid is between 1:200 (protein:lipid) to 1:30,000 (protein:lipid). In one embodiment it is about 1:10,000 (protein:lipid). In other embodiments, the ratio is about 1:2,000 or 1:4,000.

The phospholipids PC and PI have two acyl chains. The length of the acyl chains attached to the glycerol backbone varies in length from 12 to 22 carbon atoms. The acyl
15   chains may be saturated or unsaturated and may be same or different lengths. Some non-limiting examples of 12-22 carbon atom saturated and unsaturated acyl chains are shown in Tables 1A and 1B:

20

<div align="center">Table 1A</div>

Symbol	Common Name	Systematic name	Structure
12:0	Lauric acid	dodecanoic acid	$CH_3(CH_2)_{10}COOH$
14:0	Myristic acid	tetradecanoic acid	$CH_3(CH_2)_{12}COOH$
16:0	Palmitic acid	hexadecanoic acid	$CH_3(CH_2)_{14}COOH$
18:0	Stearic acid	octadecanoic acid	$CH_3(CH_2)_{16}COOH$
20:0	Arachidic acid	eicosanoic acid	$CH_3(CH_2)_{18}COOH$
22:0	Behenic acid	docosanoic acid	$CH_3(CH_2)_{20}COOH$

25

30

Table 1B

Symbol	Common Name	Systematic name	Structure
18:1	Oleic acid	9-Octadecenoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
16:1	Palmitoleic acid	9-Hexadecenoic acid	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
18:2	Linoleic acid	9,12-Octadecadienoic acid	$CH_3(CH_2)_4(CH=CHCH_2)_2(CH_2)_6COOH$
20:4	Arachidonic acid	5,8,11,14-Eicosatetraenoic acid	$CH_3(CH_2)_4(CH=CHCH_2)_4(CH_2)_2COOH$

The acyl chains attached to PC are preferably 12 to 22. These can be saturated or

5   unsaturated and can be same or different length. The acyl chains attached to PI can be from

12 to 22 and can be saturated or unsaturated. The chains of the PC and the PI can be same

or are different in length.

The PC and PI can be obtained from various sources both natural and synthetic. For

example, soy PI and egg PC are available commercially. Additionally, synthetic PC and PI

10 are also available commercially.

The compositions can be delivered by any standard route such as intravenous,

intramuscular, intraperitonial, mucosal, subcutaneous, transdermal, intradermal, oral or the

like.

15   The invention is described by the following examples, which are intended to be

illustrative and not restrictive in any way.

EXAMPLE 1

This example describes the preparation of the lipidic particles.

20 **Materials:** Albumin free full-length rFVIII (Baxter Health Care Glendale, CA) was used as

antigen. Advate was provided as a gift from Western New York Hemophilia foundation.

Dimyristoyl phosphatidylcholine (DMPC) and soybean phosphatidylinositol (Soy PI) were

purchased from Avanti Polar Lipids (Alabaster, AL). Cholesterol, IgG-free bovine serum

albumin (BSA), and diethanolamine were purchased from Sigma (St. Louis, MO). Goat

25 antimouse-Ig and antirat-Ig, alkaline phosphatase conjugates were obtained from Southern

Biotechnology Associates, Inc. (Birmingham, AL). p-Nitrophenyl phosphate disodium salt

was purchased from Pierce (Rockford, IL). Monoclonal antibodies ESH4, ESH5, and ESH8

were purchased from American Diagnostica Inc. (Greenwich, CT). Monoclonal antibody N77210M was purchased from Biodesign International (Saco, ME). Normal coagulation control plasma and FVIII-deficient plasma were purchased from Trinity Biotech (County Wicklow, Ireland). The Coamatic Factor VIII kit from DiaPharma Group (West Chester,

5   OH) was used to determine the rFVIII activity in plasma samples.

Preparation of rFVIII-PI lipidic particles: The particles were prepared by thin lipid film hydration of appropriate molar ratios of DMPC, soy PI, and cholesterol (50:50:5) with Tris buffer (TB) (25mM Tris, and 300mM NaCl, pH=7.0). The required amount of lipids was dissolved in chloroform in a kimax tube and the solvent was dried using Buchi-R200

10   rotaevaporator (Fisher Scientific, NJ). Multilamellar vesicles (MLV) were formed by vortex, mixing the lipid dispersions at 37 °C for 20 min. The resulting MLV were extruded through double polycarbonate membranes of 80nm pore size (GE Osmonics Labstore, Minnetonka, MN) in a high-pressure extruder (Mico, Inc., Middleton, WI) at a pressure of ~250 psi and then sterile-filtered through a 0.22 um MillexTM-GP filter unit (Millipore

15   Corporation, Bedford, MA). Phosphate assay was used to estimate phospholipids concentrations. Particle size was monitored using a Nicomp Model CW 380 particle size analyzer (Particle Sizing Systems, Santa Barbara, CA). The protein was added to the lipid particles at 37°C and during this process Ca^{2+} ion concentration was decreased from 5mM $CaCl_2$ to 0.2mM $CaCl_2$ in TB to ensure optimal lipid-Ca^{2+} interaction and possible lipid

20   phase change. The protein to lipid ratio was maintained at 1:10,000 for all experiments, unless stated otherwise.

Separation of Free rFVIII from rFVIII-PI: Discontinuous dextran density gradient centrifugation technique was used to separate the free protein from the lipidic particles. Briefly, 0.5 ml of incubated rFVIII-PI mixture was mixed with 1.0 ml of 20% (w/v) dextran

25   (in Ca^{+2} free TB) in a 5 ml polypropylene centrifuge tube. 3 ml of 10% (w/v) dextran was then carefully added on top of the mixture followed by 0.5 ml of Ca^{+2} free TB. After centrifugation at 45,000 rpm at 4 °C for 30min in a Beckman SW50.1 rotor, the bound protein and free lipidic particles would float to the top of the dextran band unbound rFVIII would stay at the bottom of the gradient. One-stage activated partial thromboplastin time

30   (APTT) assay was used to estimate the protein association efficiency of rFVIII-PI. This procedure yielded an association efficiency of 72 ± 9% and is much higher than that observed with Phosphatidyl Serine (PS) containing liposomes (45 ± 16.8%) (Purohit, et al. *Biochim Biophys Acta* **1617**, 31-38 (2003). Kemball-Cook, et al., *Thromb Res* **67**, 57-71 (1992)).

EXAMPLE 2

This example describes characterization of the lipid structures prepared in Example 1. The lipid dispersion samples for microscopic analysis were prepared by air-drying on formvar-coated grids and negatively staining them with 2% uranyl acetate for approximately 1 min. The samples were photographed using a Hitachi H500 TEM operating at 75 kV. Negatives were scanned at 300 dpi with an Agfa Duoscan T1200 scanner. The morphology of the particles determined using Transmission electron microscopic studies indicated the following. The particle size was found to be around 100 nm, consistent with dynamic light scattering studies (data not shown). The analysis of the micrograph showed that the donut like structures typical of liposomal lamellarity was not observed instead particles displayed disc like structures (Figure 2a) and it is possible that unique lipid organization distinct from liposomes are formed that can accommodate higher mol% of FVIII.

EXAMPLE 3

This example describes fluorescence analysis of rFVIII and rFVIII-PI. The effect of PI on the tertiary structure of rFVIII was determined by exciting the samples either at 280 nm or at 265 nm and the emission was monitored in the wavelength range of 300-400 nm. The spectra were acquired on a PTI-Quantamaster fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ). Protein concentration was 5 ug/ml and slit width was set at 4 nm. The fluorescence emission spectra of FVIII loaded in this particle showed blue shifted emission maxima compared to free protein suggesting that FVIII is located in hydrophobic environment (Figure 2b). This observation is in contrast to fluorescence spectrum observed for FVIII associated with PS containing liposomes where no change in fluorescence properties was observed (Purohit et al., 2003) and is consistent with molecular model proposed based on crystallographic and biophysical studies. FVIII was associated with PS containing liposomes only via the C-terminal region (2303-2332) of C2 domain and the rest of the molecule is accessible to bulk water (Purohit et al., 2003). However, in FVIII-PI particulates, it is likely that most of the molecular surface of FVIII is buried in hydrophobic acyl chain region of the lipidic particle and/or the protein is located at the lipid-water interface where water concentration at lipid interface may be less for PI particles.

EXAMPLE 4

This example describes Sandwich ELISA and detection of rFVIII epitopes involved in rFVIII-PI association. In order to determine the rFVIII epitopes that were associated with PI, sandwich ELISAs were performed. Briefly, Nunc-Maxisorb 96-well plates were coated

5   overnight at 4°C with appropriate concentrations of capture monoclonal antibodies in carbonate buffer (0.2 M, pH 9.6). Plates were then washed with Tween-PBS (2.7 mM KCl, 140 mM NaCl, 1.8 mM KH_2PO_4, 10 mM $Na_2HPO_4.2H_2O$, 0.05% w/v Tween 20, pH 7.4) and then blocked with 1% BSA (prepared in PBS) for 2 h at room temperature. 100 ul of 0.5 ug/ml of various dilutions of rFVIII-PI (1:0, 1:5,000, 10,000, and 50,000) or PI-containing

10   liposomes in blocking buffer were incubated at 37 °C for 1 h. Plates were washed and then incubated with 100 ul of a 1:500 dilution of rat polyclonal antibody containing a 1:1,000 dilution of goat antirat-Ig-alkaline phosphatase conjugate in blocking buffer at room temperature for 1 h. After the last wash, 200 ul of a 1 mg/ml p-nitrophenyl phosphate solution in diethanolamine buffer (1 M diethanolamine, 0.5 mM $MgCl_2$) was added and

15   incubated for 30 min at room temperature. 100 ul of 3 N NaOH were added to stop the reaction. A plate reader was used to measure the optical density at 405 nm.

In order to investigate the molecular surface area associated with PI, sandwich ELISA studies were carried out (Figure 2 c and 2d). The rationale for this experiment is that domains associated with lipidic particle are shielded and hence will not be available for

20   monoclonal antibody binding. Therefore, sandwich ELISA is an indirect, qualitative method to provide insight into protein surface accessible to bulk aqueous compartment. The binding of FVIII in the absence of PI was normalized to 100% to account for differences in binding affinity of various antibodies and decrease in antibody binding in the presence of PI was interpreted as domains of FVIII involved in PI binding. PhosphatidylCholine (PC) vesicles

25   were used as negative control as the association efficiency of FVIII in PC vesicles is around 10±4% (Purohit et al., 2003. Biochim Biophys Acta, 1617:31-38). PS liposomes were used as positive control for binding of C2 domain antibodies based on crystallographic and biophysical/biochemical studies. It has been shown that C-terminal region of the C2 domain involving 2303-2332 is involved in lipid binding and the A2 domain is further apart from

30   the liposome surface (Stoilova-McPhie et al., 2002, Blood, 99:1215-1223). Based on this molecular topology, C2 and A2 domains are spatially well separated and only lipid-binding region in C2 domain may be shielded from antibody binding due to liposome association (Purohit et al. 2003; Stoilova-McPhie et al., 2002). Monoclonal antibodies directed against C2 and A2 domains were chosen based on this molecular model of FVIII bound to PS

containing liposomes. The results indicated that the molecular surface of FVIII that is in contact with PI is different from that observed for PS. In PC liposomes no lipid concentration dependent change in antibody binding was observed indicating that no specific binding between FVIII and PC, whereas for PS vesicles, lipid concentration

5   dependent changes observed only for antibody directed against the lipid binding domain (ESH 4), consistent with the model proposed based on crystallographic studies. However, for FVIII-PI, all the monoclonal antibodies used in this study showed reduced binding and was dependent on PI concentration (Figure 2d). The results indicated that both C2 and A2 domains are somewhat inaccessible for antibody binding possibly due to steric hindrance

10   and/or substantial surface area of the FVIII molecule is buried in the PI particle.

EXAMPLE 5

This example describes CD analysis of rFVIII and rFVIII-PI. CD spectra were acquired on a JASCO-715 spectropolarimeter calibrated with d-10 camphor sulfonic acid.

15   The protein to lipid ratio was 1 to 2,500 where the protein concentration used was 20 *ug*/ml (98.6 IU/ml). Spectra were obtained over the range of 255 to 208 nm for secondary structural analysis using a 10 mm quartz cuvette. Thermal denaturation of the rFVIII and rFVIII-PI was determined by monitoring the ellipticity at 215 nm from 20 to 80 °C using a heating rate of 60 °C/h. The light scattering effect due to the presence of lipidic particles

20   was corrected as described previously (Balasubramanian et al., 2000, *Pharm Res* 17, 344-350).

The CD studies showed that such molecular topology did not alter the secondary structure of the protein, as the CD spectrum of the protein was not changed by PI association (Figure 2e). Thermal unfolding is often used to investigate the intrinsic stability.

25   FVIII associated with PI nano particle displayed a shallower melting with Tm slightly higher than that observed for FVIII indicating that the association of FVIII with PI improved the intrinsic stability of FVIII (Figure 2f).

EXAMPLE 6

30   The association of the protein was carried out in several buffer systems as shown in Tables 2A-2C. In all the cases the protein to lipid ratio was 1:10,000. The free protein was then separated from the the lipidic structures by density gradient centrifugations and the protein associated with each fraction was measured using activity and spectroscopic assay.

Table 2A shows the percentage of rFVIII associated with 50% DMPC:50% SPI:5% Chol (100nm) with different buffer compositions at 37°C. Table 2B shows the percentage of rFVIII associated with 50% DMPC:50% SPI (100nm) with different buffer compositions at 37°C. Table 2C: Percentage of rFVIII associated with 50% DMPC:50% SPI:15% Chol (100nm) with different buffer compositions at 37°C.

Table 2A

Buffer Composition	Percent Association
300mM NaCl, 25mM Tris, pH 7.4	70~80
PBS, pH 7.0	95
50mM Tris, 150mM NaCl, 3mM NaN$_3$, pH 7.4	96
25mM Tris, pH 7.4	42
10mM Hepes, pH 7.4	23

Table 2B

Buffer Composition	Percent Association
300mM NaCl, 25mM Tris, pH 7.4	67
PBS, pH 7.0	88
50mM Tris, 150mM NaCl, 3mM NaN$_3$, pH 7.4	58
10mM Hepes, pH 7.4	33

Table 2C

Buffer Composition	Percent Association
300mM NaCl, 25mM Tris, pH 7.4	66
PBS, pH 7.0	93
50mM Tris, 150mM NaCl, 3mM NaN$_3$, pH 7.4	51
10mM Hepes, pH 7.4	32

EXAMPLE 7

This example describes immunogenicity studies: Breeding pairs of hemophilia A mice (C57BL/6J) with a target deletion in exon 16 of the FVIII gene were used. A colony of hemophilia A mice was established and animals aged from 8-12 weeks were used for the in

vivo studies. Since the sex of the mice has no impact on the immune response, both male
and female mice were used for the studies.

The relative immunogenicity of free rFVIII and rFVIII-PI were determined in
hemophilia A mice. This mouse model is suitable for investigating immunogenicity of

5   FVIII as the antibody response patterns against FVIII are very similar to those observed in
hemophilic patients. 8 male and 10 female mice received 4 weekly intravenous injections
(via penile vein) and subcutaneous injections of 10 IU of FVIII (400 IU/kg), respectively. 2
weeks after the last injection, blood samples were collected in acid citrate dextrose (ACD)
buffer (85mM sodium citrate, 110mM D-glucose and 71mM citric acid) at a 10:1 (v/v) ratio

10   by cardiac puncture. Plasma was separated by centrifugation at 5,000 rpm at 4°C for 5min.
Samples were stored at -80°C immediately after centrifugation. The total titers were
determined by ELISA studies and the inhibitory titer was determined using a modified
Bethesda assay as described previously (verbruggen et al., Thromb Haemost, 1995,
73:2470251).

15   The results showed that PI reduced antibody response in Hemophilia mice (Figure
3). Animals treated with rFVIII-PI displayed significantly lower total antibody titers (Figure
3a and 3c) compared to animals treated with rFVIII alone. Titers were 2379±556 (±S.E.M;
n=10) for FVIII-PI given by sc route, compared to 13,167 ± 2042 (n=15) for animals treated
with rFVIII alone. These differences were significant at $P<0.05$. Animals treated with

20   rFVIII-PI by i.v. also showed lower mean antibody titers; for FVIII-PI antibody titers were
found to be of 3321±874 (n=8) and those treated with rFVIII had titers of 4569±1021(n=8)
and this difference was not significant. However, the inhibitory titers that abrogate the
activity of the protein reduced significantly for FVIII-PI given by both sc and iv route
(Figure 3 b and 3d). For sc administration, the inhibitory titers reduced by more than 70%.

25   For animals given FVIII alone by iv route, the inhibitory titers were 675±71 and it reduced
to 385±84 for FVIII-PI and this reduction is statistically significant at $p<0.05$. Together
these results indicate that PI containing lipidic particles not only reduced overall anti-rFVIII
antibody titers, but also lowered the titer of antibodies that abrogate the activity of the
protein.

30

EXAMPLE 8

This example describes Pharmacokinetics Studies. rFVIII or rFVIII-PI (10 IU/25g)
was administered to male hemophilia A mice as a single intravenous bolus injection via

penile vein. Blood samples were collected in syringes containing ACD buffer (10:1 v/v) at 0.08, 0.5, 1, 2, 4, 8, 16, 24, 36, and 48 h after the injections by cardiac puncture (n=3 mice/time point). Plasma was collected immediately by centrifugation (5,000 rpm, 5 min, 4 °C) and stored at -70 °C until analysis. Chromogenic assay was used to measure the activity

5 of rFVIII in plasma samples. The average values of rFVIII activities at each time point were used to compute basic pharmacokinetic parameters (half-life, MRT and area under the plasma activity curve) using a noncompartmental analysis (NCA)[20] (WinNonlin Pharsight Corporation, Mountainview, CA). The areas under the plasma activity (AUC) versus time curves from 0 to the last measurable activity time point were measured by log-linear

10 trapezoidal method. The elimination rate constant (lambda z) was estimated by log-linear regression of the terminal phase concentration. The elimination half-life ($t_{1/2}$) was calculated as ln 2/lambda z and MRT was calculated from AUMC/AUC where AUMC is the area under the curve plot of the product of concentration and time versus time.

Data was analyzed by ANOVA using Minitab (Minitab Inc., State College, PA).

15 Statistical difference ($p<0.05$) was detected by the Student independent t-test, and one-way ANOVA followed by Dunnette's post-hoc multiple comparison test. For PK studies, repeated-measures ANOVA was used to compare the profiles generated by the two treatments. Bailer-Satterthwaite method was used to compare differences in systemic exposure between the two treatments.

20 The MRT and AUC is found to be higher for FVIII-PI compared to FVIII and also showed prolonged terminal elimination phase. The circulation half-life of FVIII associated with PI lipidic particle (7.6 hrs) is higher than that observed for free FVIII (2.3 hrs). Substantial protein activity was detected after 48 hrs of injection for animals that are given FVIII-PI particles; in contrast no detectable FVIII activity was observed at 48 hrs in animals

25 that received FVIII alone (Figure 4). Further, the protein activity was detectable for only 24 hrs following administration of the PS containing liposomes (data not shown) and is due to the rapid uptake of PS liposomes by the RES. However, in the presence of PI it is possible that the cellular uptake is reduced and is consistent with the stealth like properties of PI.

30   EXAMPLE 9

This examples describes the Association efficiency for rFVIII and and a truncated version of FVIII. BDDrFVIII. The lipidic structures having FVIII or BDDrFVIII were prepared by a process as described above. The percent association for these proteins is shown below in Table 3.

Table 3

Percentage of rFVIII associated with lipidic particles of various compositions.

Protein (protein:lipid)	Composition (molar ratio)	Lipidic particle Size (nm)	Temperature (°C)	% Association
rFVIII (1:10,000)	DMPC:SPI (70:30)	200	37	30.7
rFVIII (1:10,000)	DMPC:SPI (70:30)	100	37	43.9
rFVIII (1:10,000)	DMPC:SPI (50:50)	100	37	74.6
rFVIII (1:10,000)	DMPC:SPI:Chol (70:30:5)	100	37	30.4
rFVIII (1:10,000)	DMPC:SPI:Chol (50:50:5)	100	37	81.2
rFVIII (1:10,000)	DMPC:SPI:Chol (50:50:5)	100	20	61.8
BDDrFVIII (1:10,000)	DMPC:SPI:Chol (50:50:5)	100	37	59.0
BDDrFVIII (1:10,000)	DMPC:SPI:Chol (50:50:5)	100	20	44.7
BDDrFVIII (1:20,000)	DMPC:SPI:Chol (50:50:5)	100	37	65.0
BDDrFVIII (1:20,000)	DMPC:SPI:Chol (50:50:5)	100	20	61.3

As seen above, DMPC:SPI:Cholesterol (50:50:5) formulation showed highest association efficiency than other formulations for both rFVIII and BDDrFVIII. Cholesterol is preferably included in the formulation to increase liposome stability in plasma. Size of liposome and association temperature, and lipid concentration all play important roles in association. DMPC:SPI:Cholesterol (50:50:5) formulation surprisingly has higher association for rFVIII than BDDrFVIII even though BDDrFVIII has lower Molecular weight and size. Conformational changes as a result of the B domain deletion could be responsible for a decrease in the binding affinity of BDDrFVIII towards PI containing lipidic particles.

Pharmacokinetics Studies

Male hemophilic mice (20-24g, 22-25 weeks old) were given 10IU/25g of rBDDFVIII associated with the lipidic structures (referred to as PI-BDDrFVIII) as a single i.v. bolus injection via the penile vein. Blood samples were collected 0.08, 0.5, 1, 2, 4, 8, 16, 24, 36, and 48 hr post dose by cardiac puncture (n=1/time point) and added to acid citrate dextrose (ACD) at a 10:1 (v/v) ratio. Plasma was separated by centrifugation at 5000 g for 5

min at 4°C and stored at -70°C until analysis. Plasma samples were analyzed for the activity of the protein by the chromogenic assay. The activities of BDDrFVIII determined at each time point were then utilized to estimate basic PK parameters (half-life, t1/2 and area under the plasma activity curve/exposure, AUAC)

5   The PK Normalized PK Profile for Free BDDrFVIII and PI-BDDrFVIII Formulation (n=1) is shown in Figure 5. The Area Under the Curve AUC (hr*IU/mL) or Free BDDrVIII was 955.1 and for BDDFVIII associated with lipidic structures was 1058.7.

EXAMPLE 10

10   This examples describes the preparation of PS containing liposomes that were used for comparison with the PI containing lipidic structures in other Examples. BDDrFVIII was mixed with solution containing different concentration of O –phospho –L- serine (OPLS) or phosphocholine in such a way that the final protein concentration is maintained constant at 3ug/mL. The OPLS or phosphocholine concentration vared between 0 and 100uM. Each

15   mixture is incubated for 5 minutes before subjecting to further analysis.
The intrinsic fluorescent of BDDrFVIII in the presence of increasing concentration of phospholipids head group was measured with a Quanta Master PTI instrument. The excitation was set to 285 nm and the emission was recorded at peak maximum (e.g. 330nm). The normalized fluorescence (F/F_0) data was plotted vs. [lipid] and used for for the

20   determination of the dissociation constant for lipid head group- BDDrFVIII interaction.

To monitor the aggregation process of BDDrFVIII in the presence and absence of OPLS and PC headgroup, the sample fluorescence anisotropy was measured as a function of temperature using the a Quanta Master PTI spectrofluorometer equipped with motorized polarizer prisms . The data was plotted as anisotropy vs. temperature and fitted to a

25   sigmoidal curve. The inflection point of each curve was obtained.

The dissociation constant (Kd (uM)) for OPLS was 70.2 and that for phophocholine was 24.2. The inflection point for free BDDrFVIII was 71.6, for BDDrFVIII in OPLS liposomes was 79.4 and for BDDrFVIII in phosphocholine liposomes was 72.2.

To study the immunological properties of BDDrFVIII- OPLS complex, 8 to 12 week

30   old hemophilic mice received 4 weekly injection containing different BDDrFVIII formulations (free BDDrFVIII anf BDDrFVIII- OPLS complex -- prepared as described above.; [OPLS] = 10mM). The dose for each injection is of 10 IU / animal. Two weeks following the last injection, blood samples were collected and analyzed for the presence of inhibitory antibodies using a modified Bethesda assay. Results indicated a statistical

significant decrease in the immune response for the BDDrFVIII – OPLS complex compared to free BDDrFVIII (P<0.05).

The association efficiency for rFVIII and BDDrFVIII – phosphatidylserine – containing liposomes was determined. Dimyristoylphosphatidylcholine (DMPC): brain phosphatidylserine (BPS) liposomes (molar ratio 70:30). The required amounts of DMPC and BPS were dissolved in chloroform. A thin lipid film was formed on the walls of a glass tube, by removing the solvent in a Buchi-R200 rotoevaporator (Fisher Scientific). The liposomes were prepared by rehydration of the lipid film with Tris buffer (TB 25mm Tris, 300 mM NaCl, 5mM $CaCl_2$ pH =7.4) at 37°C. The liposomes were extruded eight times through double stacked 100nm or 200 nm polycarbonate membranes using a high pressure extruder (Lipex Biomembranes, Inc.) at a pressure of ~200 psi. The size distribution of the liposomes was monitored using a Nicomp model CW380 size analyzer (Particle Sizing System).

Liposomal protein preparation

The association of the protein with the preformed liposomes was achieved by incubating the protein in the presence of the liposomes at 37°C for 30 minutes with occasional gentle swirling. The protein to molar ratio was maintained the same for all preparation (1:10,000).

PEGylation of preformed protein-liposome mixtures.

PEGylation of the preformed liposomes was achieved by addition of the liposomal preparations to a dry powder of 1,2 dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylenglycol) 2000] (DMPC-PEG 2000) or 1,2 distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylenglycol) 2000] (DSPE-PEG 2000). The incubation was performed for 45 minutes at room temperature. Care was taken to maintain the DMPC-PEG 2000 concentration below the critical micellar concentration in order to facilitate the transfer of DMPC-PEG 2000 to the preformed lipidic bilayer.

Separation of Free protein from Liposome Associated protein

To estimate the amount of protein associated with liposomes, free protein was separated from liposome-associated protein by floatation on a discontinuous dextran density gradient. Briefly, 0.5ml of the liposome-protein mixture was mixed with 1ml of 20% (w/v) dextran (in calcium free Tris buffer) in a 5ml polypropylene centrifuge tube and 3ml of 10% (w/v) dextran and 0.5ml calcium free Tris buffer were overlaid on the liposome-containing band. The gradient was subjected to ultracentrifugation at 45,000rpm for 30min in a Beckman SW50.1 rotor. The liposomes and their associated protein floated to the interface of the buffer/10% dextran bands, and the unassociated protein remained at the bottom. The

activity of the protein associated with liposomes was determined using the one-stage

activated partial thromboplastin time (APTT) assay. Results are shown in Table 4A and 4B.

Table 4A

Association efficiency % Non- PEGylated liposomes				
BDDrFVIII	200nm	DMPC:BPS	(70:30)	41.5
BDDrFVIII	100nm	DMPC:BPS	(70:30)	44.4

Table 4B

PEGylated liposomes				
rFVIII (Control)	200nm	DMPC:BPS	(70:30)	28.0
BDDrFVIII	200nm	DMPC:BPS	(70:30)	38.0
BDDrFVIII	100nm	DMPC:BPS	(70:30)	48.3

BDDrFVIII retains all critical structural characteristics of the parent molecule, including the binding properties towards phosphatidylserine (PS) containing lipidic membranes as well as its activity. The association efficiency of BDDrFVIII was higher than that observed for full length rFVIII.

Immunological properties of BDDrFVIII- PS containing liposomes were also tested. Eight to 12 week old hemophilic mice received 4 weekly injections containing 10 IU of BDDrFVIII – PS containing liposomes (prepared as described in example 3). Two weeks following the last injection, blood samples were collected and analyzed for the presence of inhibitory antibodies using a modified Bethesda assay.

EXAMPLE 11

This example describes the association efficiency for BDDrFVIII associated with PS and phosphatidylethanolamine (PE) containing liposomes which were used for comparison purposes.

Liposome preparation.

DMPC: BPS: dioleoylphosphatidylethanolamine (DOPE) (molar ratio 70:10:20) were prepared as described bellow: The required amounts of DMPC, BPS and DOPE were dissolved in chloroform. A thin lipid film was formed on the walls of a glass tube, by

removing the solvent in a Buchi-R200 rotoevaporator (Fisher Scientific). The liposomes

were prepared by rehydration of the lipid film with Tris buffer (TB 25mm Tris, 300 mM

NaCl, 5mM $CaCl_2$ pH =7.4) at 37°C. The liposomes were extruded eight times through

double stacked 100nm polycarbonate membranes using a high pressure extruder (Lipex

5   Biomembranes, Inc.) at a pressure of ~200 psi. The size distribution of the particles was

monitored using a Nicomp model CW380 size analyzer (Particle Sizing System).

Liposomal protein preparation

The association of the protein with the preformed liposomes was achieved by incubating the

protein in the presence of the liposomes at 37°C for 30 minutes with occasional gentle

10   swirling. The protein to molar ratio was maintained the same for all preparation (1:10,000).

PEGylation of preformed protein-liposome mixtures.

PEGylation of the preformed liposomes was achieved by addition of the liposomal

preparations to a dry powder of 1,2 dimyristoyl-sn-glycero-3-phosphoethanolamine-N-

[methoxy (polyethylenglycol) 2000] (DMPC-PEG 2000) or 1,2 distearoyl-sn-glycero-3-

15   phosphoethanolamine-N-[methoxy (polyethylenglycol) 2000] (DSPE-PEG 2000). The

incubation was performed for 45 minutes at room temperature. Care was taken to maintain

the DMPC-PEG 2000 concentration below the critical micellar concentration in order to

facilitate the transfer of DMPC-PEG 2000 to the preformed lipidic bilayer.

Separation of Free protein from Liposome Associated protein

20   To estimate the amount of protein associated with liposomes, free protein was

separated from liposome-associated protein by floatation on a discontinuous dextran density

gradient. Briefly, 0.5ml of the liposome-protein mixture was mixed with 1ml of 20% (w/v)

dextran (in calcium free Tris buffer) in a 5ml polypropylene centrifuge tube and 3ml of 10%

(w/v) dextran and 0.5ml calcium free Tris buffer were overlaid on the liposome-containing

25   band. The gradient was subjected to ultracentrifugation at 45,000rpm for 30min in a

Beckman SW50.1 rotor. The liposomes and their associated protein floated to the interface

of the buffer/10% dextran bands, and the unassociated protein remained at the bottom. The

activity of the protein associated with liposomes was determined using the one-stage

activated partial thromboplastin time (APTT) assay. Results are shown in Table 5.

30

Table 5

Exp #		size	composition		Percent association
I	Control	100nm	DMPC:BPS:DOPE	(90:10:00)	18.0
II		100nm	DMPC:BPS:DOPE	(70:10:20)	57.4
III		100nm	DMPC:BPS	(70:30)	44.4
IV		100nm	DMPC:BPS:DOPE	(70:10:20) +3% PEG	45.6

PS containing liposomes are rapidly cleared from circulation by the reticuloendothelium system (RES). Phopsphatidylethanolamine (PE) increases the affinity of FVIII towards PS containing lipids. In the present example, PE is added to the composition at the expense of PS. The association efficiency is found to increase with increasing concentration of PE (compare Exp # I and II). In the absence of PEG, the association efficiency is found to be higher for DOPE containing particles than in the absence of DOPE (Exp # II and III). Decreasing the content of PS in the formulation while achieving a higher association efficiency is more beneficial from the perspective of pharmacological properties of BDDrFVIII.

EXAMPLE 12

This example describes the application of this method to another protein, Factor VII. In this example, lipidic structures comprising FVII were prepared. The required amounts of DMPC, SPI and Chol (Dimyristoylphosphatidylcholine (DMPC): soy phosphatidylinositol (SPI): Cholesterol (Chol) (molar ratio 50:50:5) were dissolved in chloroform. A thin lipid film was formed on the walls of a glass tube, by removing the solvent in a Buchi-R200 rotoevaporator (Fisher Scientific). The lipidic particles (LP) were prepared by rehydration of the lipid film with 25 mM tris buffer (300 mM NaCl, pH =7.0; calcium free) at 37°C. The LP were extruded twenty times through double stacked 80 nm polycarbonate membranes using a high pressure extruder (Lipex Biomembranes, Inc.) at a pressure of ~200 psi. The size distribution of the particles was monitored using a Nicomp model CW380 size analyzer (Particle Sizing System).

The association of the protein with the LP was achieved by incubating the protein in the presence of the LP at 37°C for 30 minutes. The protein to lipid molar ratio was maintained for the first two trials of preparation (1:10,000). Additional, one trial using protein:lipid ratios of 1:2000 were also investigated.

To estimate the amount of protein associated with LP, free protein was separated from LP-associated protein by floatation on a discontinuous dextran density gradient. Briefly, 0.5ml of the LP-protein mixture was mixed with 1ml of 20% (w/v) dextran (in calcium free Tris buffer) in a 5ml polypropylene centrifuge tube and 3ml of 10%

5 (w/v) dextran and 0.5ml calcium free Tris buffer were overlaid on the LP-containing band. The gradient was subjected to ultracentrifugation at 45,000rpm for 30min in a Beckman SW50.1 rotor. The LP and their associated protein floated to the interface of the buffer/10% dextran bands, and the unassociated protein remained at the bottom. The concentration of the protein associated with LP was determined using spectroscopic assay. The percent

10 association for protein:lipid of 1:10,000 was 63.9±9.6% (n=3) and the percent association for protein:lipid of 1:2,000 was 40.1±1.1% (n=3).

EXAMPLE 13

This example describes the application of this method to another protein, lysozyme.

15 The required amounts of DMPC, SPI and Chol (Dimyristoylphosphatidylcholine (DMPC): soy phosphatidylinositol (SPI): Cholesterol (Chol) (molar ratio 50:50:5) were dissolved in chloroform. A thin lipid film was formed on the walls of a glass tube, by removing the solvent in a Buchi-R200 rotoevaporator (Fisher Scientific). The lipidic particles (LP) were prepared by rehydration of the lipid film with 25 mM tris buffer (300 mM NaCl, pH =7.0;

20 calcium free) at 37°C. The LP were extruded twenty times through double stacked 80 nm polycarbonate membranes using a high pressure extruder (Lipex Biomembranes, Inc.) at a pressure of ~200 psi. The size distribution of the particles was monitored using a Nicomp model CW380 size analyzer (Particle Sizing System).

The association of the protein with the LP was achieved by incubating lysozyme in

25 the presence of the LP at 37°C for 30 minutes. The protein to lipid molar ratio maintained at 1:2000 was investigated.

To estimate the amount of protein associated with LP, free protein was separated from LP-associated protein by floatation on a discontinuous dextran density gradient. Briefly, 0.5ml of the LP-protein mixture was mixed with 1ml of 20% (w/v) dextran (in

30 calcium free Tris buffer) in a 5ml polypropylene centrifuge tube and 3ml of 10% (w/v) dextran and 0.5ml calcium free Tris buffer were overlaid on the LP-containing band. The gradient was subjected to ultracentrifugation at 45,000rpm for 30min in a Beckman SW50.1 rotor. The LP and their associated protein floated to the interface of the buffer/10% dextran

bands, and the unassociated protein remained at the bottom. The concentration of the
protein associated with LP was determined using spectroscopic assay.

Protein: Lipid	Composition (molar ratio)	% Association
1:2,000	DMPC (100)	47.5
1:2,000	DMPC:SPI:Chol (50:50:5)	81.9

5   The particle thus prepared packaged the protein inside the particle and shields from
the surrounding milieu. This is supported by acrylamide quenching data as shown in Figure
6. This is likely to provide in vivo stability as it may shield the protein from protease
degradation.

10   EXAMPLE 14

     This example describes the association of another protein, erythropoietin (EPO) with
the lipidic particles of the present invention. DMPC, SPI and Chol
(Dimyristoylphosphatidylcholine (DMPC): soy phosphatidylinositol (SPI): Cholesterol
(Chol) (molar ratio 50:50:5) were dissolved in chloroform. A thin lipid film was formed on
15 the walls of a glass tube, by removing the solvent in a Buchi-R200 rotoevaporator (Fisher
Scientific). The lipidic particles (LP) were prepared by rehydration of the lipid film with
25mM tris buffer (300mM NaCl, pH = 7.0, calcium free) at 37°C. The LP were extruded
twenty times through double stacked 80 nm polycarbonate membranes using a high pressure
extruder (Lipex Biomembranes, Inc.) at a pressure of ~200 psi. The size distribution of the
20 particles was monitored using a Nicomp model CW380 size analyzer (Particle Sizing
System).

     The association of the protein erythropoietin with the LP was achieved by
incubating EPO in the presence of the LP at 37°C for 30 minutes. The protein to lipid molar
ratio was maintained as 1:10,000 (3 trials) and 1:2000 (1 trial).

25   To estimate the amount of protein associated with LP, free protein was separated
from LP-associated protein by floatation on a discontinuous dextran density gradient.
Briefly, 0.5ml of the LP-protein mixture was mixed with 1ml of 20% (w/v) dextran (in
calcium free Tris buffer) in a 5ml polypropylene centrifuge tube and 3ml of 10% (w/v)
dextran and 0.5ml calcium free Tris buffer were overlaid on the LP-containing band. The
30 gradient was subjected to ultracentrifugation at 45,000rpm for 30min in a Beckman SW50.1

rotor. The LP and their associated protein floated to the interface of the buffer/10% dextran bands, and the unassociated protein remained at the bottom. The concentration of the protein associated with LP was determined using spectroscopic assay.

5   The association efficiency as determined by fluorescence spectroscopy was 74.90%, 68.60% and 68.50% for a protein:lipid ratio of 1:10,000 and 51% for a protein;lipid ratio of 1:2,000.

While the invention has been described through specific examples, routine modifications will be apparent to those skilled in the art and such modifications are intended 10   to be within the scope of the invention..

Claims:

1.   Lipidic particles comprising phophatidylcholine, phophatidylionositol and cholesterol, wherein the ratio of PC to PI is between 70:30 to 30 to 70 and cholesterol is present between 1 to 33% of PC and PI together, the particles have a size of between 40 to 140 nm.

2.   The particles of claim 1, wherein the PC to PI ratio is between 60:40 to 40:60.

3.   The particles of claim 2, wherein the PC to PI ratio is between 55:45 and 45:55.

4.   The particles of claim 3, wherein the PC to PI ratio is 50:50 and cholesterol is between 5-15% of PC and PI together.

5.   The particles of claim 1, wherein each acyl chains of PC and PI independently has between 12 and 22 carbon atoms, and is saturated or unsaturated.

6.   The particles of claim 1, wherein the particles are present in a lyophilized form.

7.   The particles of claim 1, wherein the PI is soy PI and PC is egg PC.

8.   A composition comprising the lipidic particles of claim 1, and further comprising one or more therapeutic agents associated therewith such that the immunogenicity of the therapeutic agent is reduced, wherein the therapeutic agent is a peptide, polypeptide or a protein.

9.   The composition of claim 8, wherein the therapeutic agent is a protein involved in the blood coagulation cascade.

10.   The composition of claim 9, wherein the therapeutic agent is selected from the group consisting of factor VIII, Factor VII, Factor IX, Factor V, Willebrand Factor (vWF) and von Heldebrant Factor (vHF).

11.   The composition of claim 8 wherein the therapeutic agent is selected from the group consisting of tissue plasminogen activator, insulin, growth hormone, erythropoietin alpha, VEG-F, thrombopoietin and lysozyme.

5

12.   The composition of claim 8, wherein at least 50% of the lipidic particles have a size of 140 nm or less.

13.   The composition of claim 12, wherein at least 60%, 70% 80% or 90% of the
10   lipidic particles have a size of 140 nm or less.

14.   The composition of claim 12, wherein at least 50%, 60%, 70%,80% or 90% of the lipidic particles have a size between 40 to 100 nm.

15   15.   The composition of claim 12, wherein the size of the lipidic particles is between 80 and 100 nm.

16.   A method of reducing the immunogenicity of a therapeutic agent selected from the group consisting of a peptide, polypeptide or a protein comprising the steps
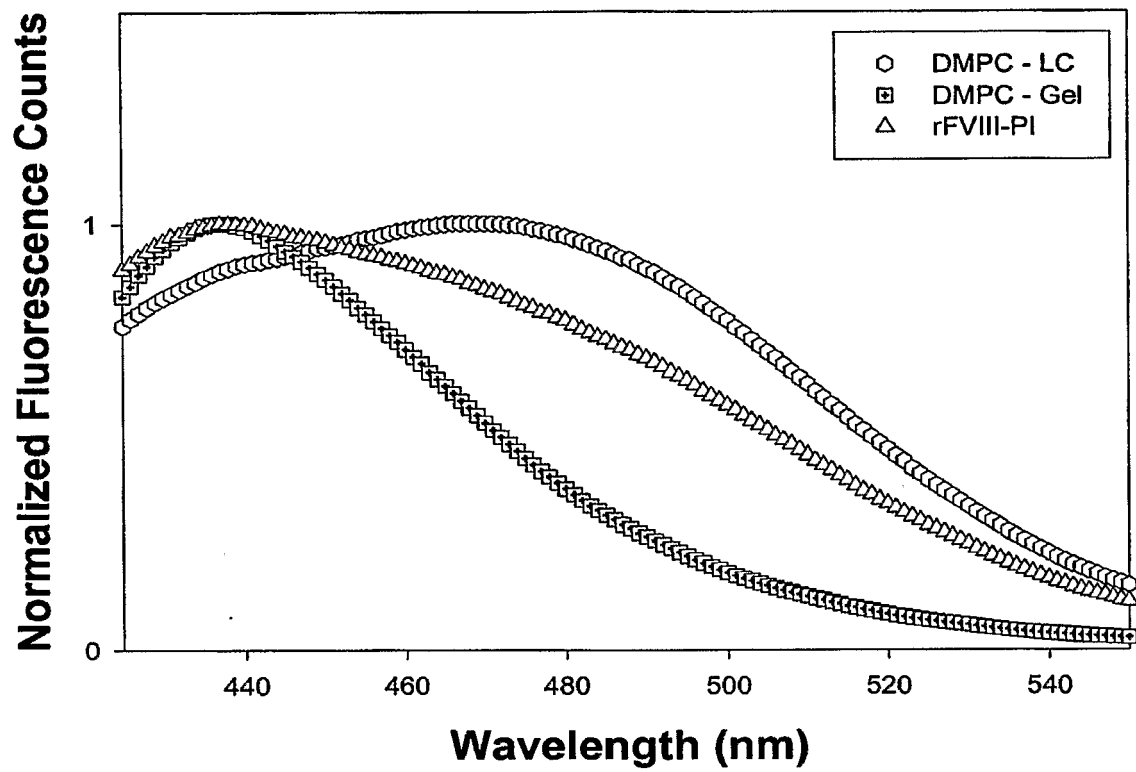20   of:

        a) preparing the lipidic particles of claim 1 by extruding multilamellar vesicles comprsing PC, PI and cholesterol through a sizing device to form lipidic particles of less than 140 nm; and

        b) mixing the therapeutic agent with the lipidic particles prepared in step a)
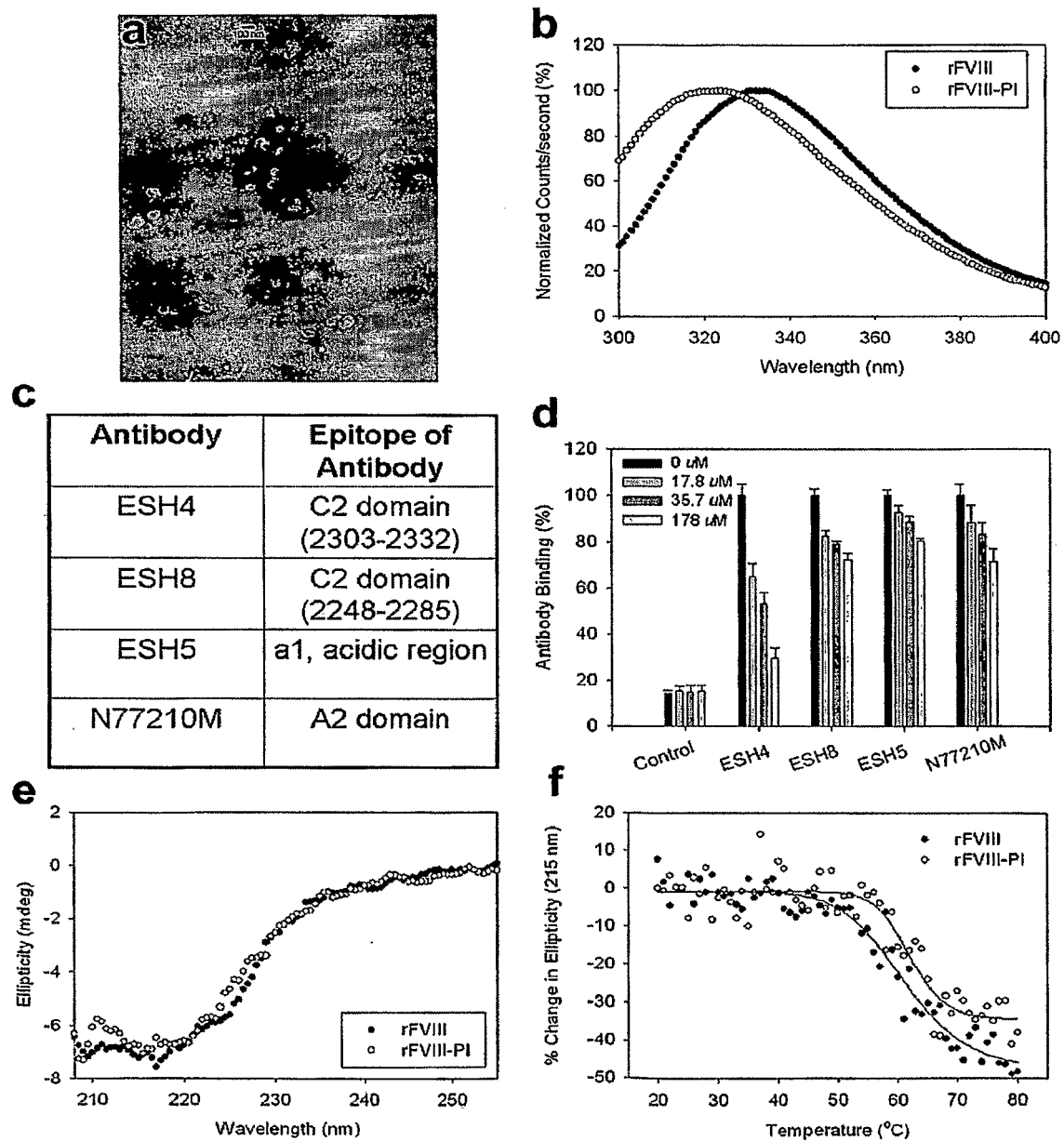25   wherein the immunogenicity of the therapeutic agent is reduced.
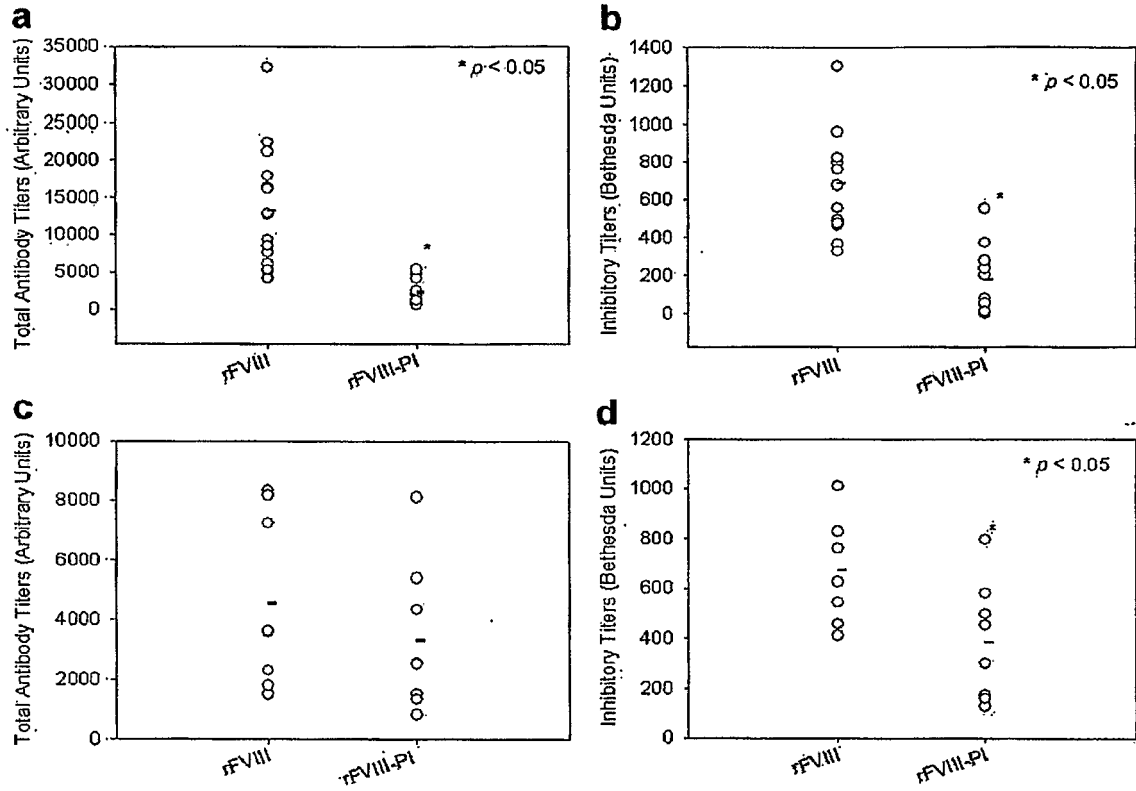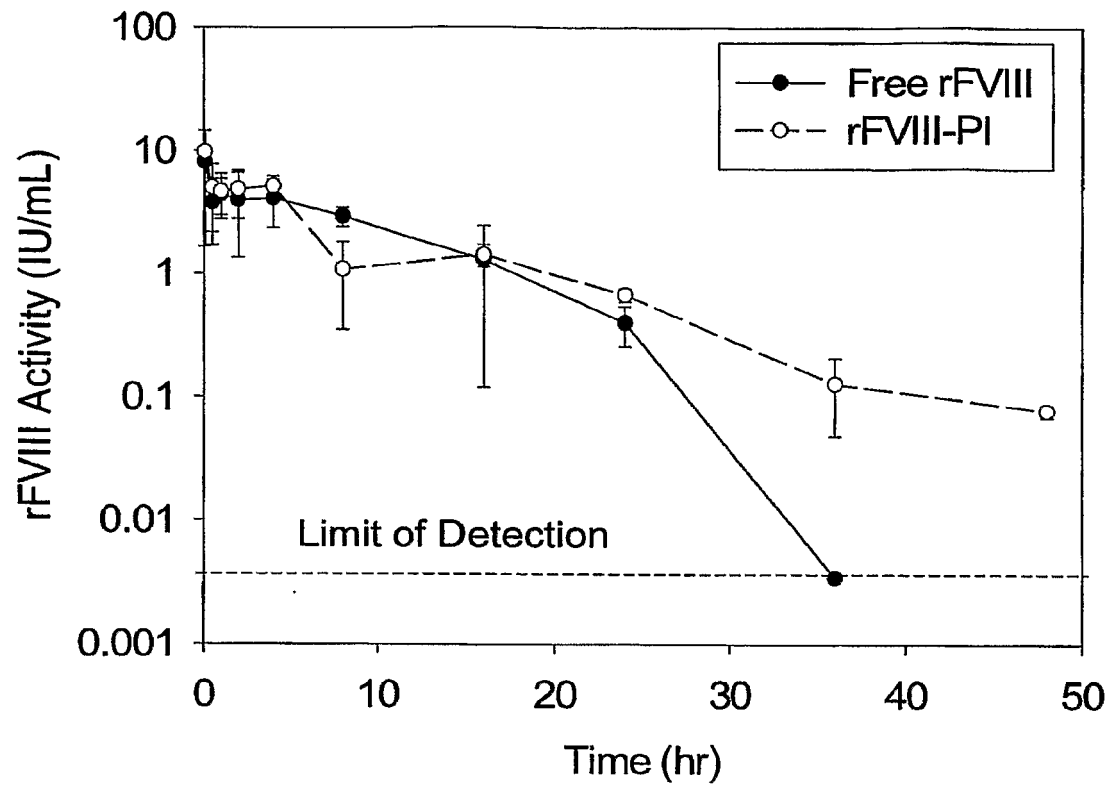
5

Figure 1

10

Figure 2

Antibody	Epitope of Antibody
ESH4	C2 domain (2303-2332)
ESH8	C2 domain (2248-2285)
ESH5	a1, acidic region
N77210M	A2 domain

Figure 3

Figure 4

5

Figure 5

Figure 6