



(86) Date de dépôt PCT/PCT Filing Date: 2006/02/02
(87) Date publication PCT/PCT Publication Date: 2006/08/10
(85) Entrée phase nationale/National Entry: 2007/08/01
(86) N° demande PCT/PCT Application No.: US 2006/003779
(87) N° publication PCT/PCT Publication No.: 2006/084095
(30) Priorité/Priority: 2005/02/02 (US11/049,134)

(51) Cl.Int./Int.Cl. *A61K 35/14* (2006.01)
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(54) Titre : COMPOSITIONS ET METHODES D'OBTENTION DE PREPARATIONS MOINS IMMUNOGENES
(54) Title: COMPOSITIONS AND METHODS FOR LESS IMMUNOGENIC PROTEIN FORMULATIONS

(57) **Abrégé/Abstract:**

Methods and composition are described for low immunogenic protein, polypeptide or peptide formulations. The method comprises forming complexes of the protein, polypeptide or peptide with a binding agent which can be a serine containing compound or other phosphatidyl compounds or phospholipids. The complexes formed include simple complexes, micelles, cochleate structures, liposomes, non-bilayer and novel lipidic structures. The antigenicity and imminogenicity of the protein, polypeptides or peptides is reduced upon formation of such complexes.



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

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 August 2006 (10.08.2006)

PCT

(10) International Publication Number
WO 2006/084095 A1

(51) International Patent Classification:
A61K 35/14 (2006.01)

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(21) International Application Number:
PCT/US2006/003779

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

(22) International Filing Date: 2 February 2006 (02.02.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/049,134 2 February 2005 (02.02.2005) US

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

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Published:

- *with international search report*
- *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMPOSITIONS AND METHODS FOR LESS IMMUNOGENIC PROTEIN FORMULATIONS

(57) Abstract: Methods and composition are described for low immunogenic protein, polypeptide or peptide formulations. The method comprises forming complexes of the protein, polypeptide or peptide with a binding agent which can be a serine containing compound or other phosphatidyl compounds or phospholipids. The complexes formed include simple complexes, micelles, cochleate structures, liposomes, non-bilayer and novel lipidic structures. The antigenicity and imminogenicity of the protein, polypeptides or peptides is reduced upon formation of such complexes.

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COMPOSITIONS AND METHODS FOR LESS IMMUNOGENIC PROTEIN FORMULATIONS

5 This invention was made with Government support under grant no. R01 HL-70227-01 from the National Institutes of Health. The Government has certain rights in the invention.

FIELD OF THE INVENTION

10 The present invention relates to protein complexes having low immunogenicity and a method of making same.

DISCUSSION OF RELATED ART

15 Hemophilia is a bleeding disorder caused by the deficiency of factor VIII (anti hemophilic factor, AHF or FVIII). FVIII is a multi domain protein comprising of six domains A1, A2, A3, B, C1 and C2 and activation of this protein by thrombin results in heavy (A1 and A2) and light chain (A3, C1 and C2) [1, 2]. Replacement therapy using blood concentrate, recombinant factor VIII and variants of factor VIII is the first line therapy for hemophilia. However, 15-35% of patients develop neutralizing antibodies
20 and such immune response compromises therapy for hemophilia. Current treatment regimens to overcome neutralizing antibody development exist but are not cost effective. Development of less immunogenic Factor VIII preparations could offer an alternate clinical approach.

25 In general, it has been shown that the immune response to a therapeutic protein is due to the following reasons; (i) route of administration, (ii) existence of aggregates, (iii) frequent administration and (iv) specific epitope regions [3]. Recently, it has been shown that FVIII has a tendency to form aggregates [4] and the role of these aggregates in the development of immune response is not well understood. Further, there are primarily two epitope regions on FVIII molecule, C2 and A2 domains. Scandella et al
30 [5] has shown that the antibody titre is highest against the C2 domain of the light chain. The C2 domain is also a membrane binding domain and it binds to phosphatidyl serine (PS) on platelet membranes as part of its coagulation cascade [6,7]. The anticoagulant action of antibodies to the C2 domain is due to inhibition of binding of factor VIII to phospholipid. It has been shown that the monoclonal antibodies against the C2 domain

prevent the binding of Factor VIII to phospholipid containing membranes and based on these observations it was concluded that the epitope and membrane binding regions overlap [10, 11].

Previous studies on liposomal encapsulation of FVIII were aimed at increasing the encapsulation efficiency of FVIII (Factor VIII) in conventional liposomes and further to increase the in vivo stability and oral bioavailability [12, 13]. Due to the molecular architecture, lipid molecules tend to form several molecular assemblies such as liposomes, micelles, non-bilayer structures and cochleate structures. Extensive studies have been done to use these molecular assemblies as drug delivery vehicles to improve the therapeutic properties of several drugs including proteins and peptides. These therapeutic properties are prolonging circulation time, reducing the toxicity, enhancement of immune response and reduction of in vivo degradation [14-25]. Thus, liposomes have been used as adjuvants to increase the immune response [26-31]. However, development of lipid complex to reduce immune response and antigenicity has not been investigated and therefore, there continues to be a need to develop approaches for reducing the immunogenicity of therapeutic proteins. Another approach has been to modify the sequence of the specific epitopes to reduce immuno toxicity [32]. However, such amino acid substitutions could lead to loss of biological activity.

SUMMARY OF THE INVENTION

The present invention discloses compositions having low antigenicity and immunogenicity and methods of making same. Accordingly, compositions comprising a therapeutic agent such as a protein, polypeptide or peptide and one or more molecules capable of binding to the protein (referred to herein as the binding agent) in such a way as to reduce its immunogenicity and antigenicity are disclosed. Such binding agents include serine compounds such as phosphoserine, phosphatidyl serine, or phospholipids comprising phosphatidyl serine (PS); phosphatidyl choline (PC), phosphatidic acid (PA), or phosphoethanolamine (PE); or phospholipids containing PA, PC, or PE.

The protein-binding agent complexes can be in the form of (1) liquid or freeze dried form of this liquid containing protein-binding agent complex (2) novel non-liposomal structures, (3) liposomes (4) micelles (5) cochleate (6) non-bilayer structures which reduce the immune response.

The present invention also discloses a method for reducing the immunogenicity

and/or antigenicity of a protein by forming a complex with a binding agent (such as a serine containing compound). The protein-binding agent complex may be stabilized with suitable buffers.

In one embodiment, the dried lipid film containing dimyristyl phosphatidyl choline (DMPC) and brain phosphatidyl serine (bPS) is hydrated using protein (such as FVIII) in various buffer systems. Novel, non liposomal structures are formed using DMPC, bPS in 300mM NaCl and 5mM CaCl₂. Conventional liposomes are formed as the buffer system is changed to water or phosphate buffered saline. This can also be accomplished by reducing the calcium or PS concentrations. The removal of DMPC and using 100% PS and a sonication or extrusion step leads to cochleate structures and use of PS with intermediate acyl chain length results in micellar structures. Use of shorter acyl chain length at lower concentrations yield protein-lipid complexes in solution.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the melting profile of FVIII at different heating rates.

Figure 2 is the antibody binding assay that shows the conformational changes in the C2 domain. Binding of monoclonal antibodies ESH 4 and ESH8 to rFVIII as determined by sandwich ELISA following the heating of rFVIII at 60°C and 15°C/hr to the indicated temperatures.

Figure 3 is a representation of size exclusion chromatography (SEC) profiles of Factor VIII in the presence or absence of O-Phospho-L-Serine.

Figure 4 is a representation of antigenicity of FVIII-O-Phospho-L-Serine studied by sandwich ELISA.

Figure 5A and 5B are representation of the effect of OPLS, phosphocholine and phosphatidic acid on the immunogenicity of rFVIII. Average total antibody titres (5A) and inhibitory titres (5B) are shown for the indicated binding agent-rFVIII complexes compared to rFVIII.

Figure 6 is a representation of folding studies of FVIII in O phospho L-Serine.

Figure 7 is a representation of antigenicity of FVIII-PS complex in liposomes studied by sandwich ELISA. FVIII: Free FVIII, Invention FVIII: Composition used in the present invention and DMPC+FVIII: Physical Mixture of DMPC liposomes and FVIII lacking specific protein (FVIII) lipid (PS) complex.

Figure 8A is a representation of the immune response in animal models for free FVIII and FVIII-PS complex. FVIII: Free FVIII, Invention FVIII: Composition used in the present invention.

Figure 8B is a representation of FVIII and FVIII-PS complex liposomes in Factor VIII knockout Hemophilia A mice model.

Figure 9 is the photograph of a Dextran density gradient showing the non-liposomal, low water volume fraction containing FVIII-PS complex.

Figure 10 is a representation of the effect of DCPS on the antigenicity of rFVIII

10 DETAILED DESCRIPTION OF THE INVENTION

The terms AHF, Factor VIII and FVIII are used interchangeably to mean the same molecule.

The present invention provides a method for reducing the antigenicity and immunogenicity of proteins. While the term "protein" is used throughout the application, it is intended to include peptides (generally considered to be 50 or less amino acids) as well as polypeptides (generally considered to be more than 50 amino acids).

The method of the present invention comprises the steps of forming complexes of one or more proteins, polypeptides or peptides with a phospholipid, preferably a phospholipid containing serine. Various types of protein-lipid structures can be formed depending upon the particular phospholipid, concentration and combinations of phospholipids

In general, the term "Liposome" means a generally spherical or spheroidal cluster or aggregate of lipid compounds, typically in the form of one or more concentric layers, for example, monolayers, bilayers or multi-layers. They may also be referred to as lipid vesicles. The liposomes may be formulated, for example, from ionic lipids and/or non-ionic lipids.

The terms "Cochleates" or "cochleate structures" generally refer to a multilamellar lipid vesicle that is generally in the shape of a spiral or a tubule.

The term "Micelles" refers to colloidal entities formulated from lipids. Micelles may comprise a monolayer, bilayer, or hexagonal phase structure.

In the present invention it was observed that different structures were formed with the protein/polypeptide complexes depending upon the binding agent. In general,

if serine containing agents were used, simple complexes, micelles, liposomes, cochleate structures and novel condensate phase were observed. If other phosphatidyl compounds or phospholipids were used (such as PC, PG, PA or PE or phospholipids containing PC, PG or PE), only micelles or liposomes were observed. Further, only PE
5 containing phospholipids were observed to form bilayer and non-bilayer (hexagonal) structures .

The use of phosphoserine, phosphatidyl serine, or PS containing phospholipids with short acyl chain length (i.e, with 4 or less acyl chain carbon atoms) did not lead to the formation of lipid molecular assemblies. The structures formed are simple
10 complexes. These simple complexes are characterized by mostly ionic bonding. Use of serine containing phospholipids having intermediate acyl chain length (i.e., between 5-12 acyl chain carbon atoms) above its critical micellar concentration, form micelles and below critical micellar concentration form simple complexes. The phospholipids having longer acyl chain length (12-18 carbon atoms), due to the molecular architecture, tend
15 to form several molecular assemblies such as liposomes, non-bilayer structures and cochleate structures.

The use of other phosphatidyl compounds such as PC, PG, PA and PE and phospholipids containing PC, PG, PA and PE results in the formation of micelles for intermediate length acyl chain carbon atoms and liposomes for longer length acyl chain
20 carbon atoms.

In all the phospholipids the acyl chain may be a diacyl chain or a single acyl chain. The term phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl glycerol means there is no acyl chain in the compound.

The lipid composition may be varied to prepare liposome, non-bilayer structures and cochleate phases. A lipid composition of phosphatidyl choline (PC): phosphatidyl
25 serine (PS) or phosphoserine with high PC content will form liposomes upon hydration with buffers containing Ca^{2+} and Na^+ . The presence of Phosphatidyl Ethanolamine PE and PS promote the formation of non-bilayer structures. The formulation with PS (over 90mol%) in the presence of Ca^{2+} with no or lower Na^+ (100mM) promote the formation
30 of cochleate cylinders. Conditions such as temperature, $\text{Ca}^{2+}/\text{Na}^{2+}$ can be altered to reduce the size of cochleate cylinders in the nano-particles containing protein-lipid complex.

The phospholipids useful for the present invention include serine containing

compounds including phospholipids. Examples of such serine containing phospholipids are O-Phospho-L-Serine (OPLS), Dicaproyl Phosphatidyl Serine, Dioctanoyl Phosphatidyl Serine, Dimyristoyl, Dipalmitoyl, Dioleoyl-, Distearoyl-Phosphatidyl Serine. The serine containing phospholipids may be used in combination with other molecules including other phospholipids. For example, phosphatidyl serine can be used in combination with phosphatidyl choline or Phosphatidyl ethanolamine. In one embodiment, the PS, PC, PA and PE are acylated or diacylated. The phosphatidyl serine may be obtained from any source such as natural (brain) or from synthetic origin. Phosphatidyl serine may be used in combination with phosphatidyl choline (PC). The phosphatidyl choline may be dimyristoyl phosphatidyl choline. The ratio of the PS and PC can be varied from 1:9 to 9:1. In one embodiment, the ratio is 3:7.

The protein-lipid compositions of the present invention are preferably stabilized and stored in suitable buffer systems. Such buffers include TRIS buffer and HEPES buffer and sodium and calcium salts. Optionally alcohol (such as 10% ethanol) may be added.

The protein-lipid complexes of the present invention can be characterized by standard methods. For example, fluorescence studies can be carried out on a SLM AMINCO 8000 series instrument or PTI 380 instrument using 4nm as excitation and emission slits. The samples can be excited at 280 nm and the emission spectra scanned in the range of 300 to 400 nm. The emission spectra of free Factor VIII was observed around 335 nm and the addition of OPLS reduced the intensity of fluorescence emission indicating that the tertiary structure of the protein is altered slightly.

Further, the particle size of the lipid associated protein can be determined using a standard particle sizer (such as NICOMP 315 model). The particle size distribution can be analyzed using both Gaussian and NICOMP analysis for unimodal and bimodal distribution. The size of latex beads can be used as standard controls with each measurement.

The lipid structures can also be analyzed by negative staining electron microscopy. Such methods are routine in the art and can be used to confirm that there are no aggregates and to classify the structures as liposomes, non-bilayers or cochleates. The formation of non-bilayer and cochleate structures can also be investigated using Laurdan fluorescence. The lipid structures can be labeled with the

probe by mixing the lipid containing solution with aqueous solution of the probe (containing 0.01% ethanol). The samples can be excited at 340 nm and the emission spectra were monitored at 440 nm. The excitation spectra can be acquired in the range of 320 and 420 nm, with emission monochromator at 440 nm.

- 5 The protein lipid complexes of the present invention can be delivered to an individual (such as an animal including a human being) by any standard means of administration such as intramuscular, intranasal, intraperitoneal, intravenous, oral, rectal, subcutaneous, topical and the like. The complexes may be delivered directly to or near the target site or may be delivered directly or indirectly into the circulation.
- 10 The complexes may be delivered in pharmaceutically acceptable carriers which are well known in the art.

- The protein-lipid complexes of the present invention exhibit reduced immunogenicity as well as reduced antigenicity. Accordingly, such compositions can be used for reducing immune response in an individual against a therapeutic agent. The
- 15 compositions of the present invention can also be used for delivery of a therapeutic agent to an individual in whom an immune reaction to the protein has already occurred. Thus, these composition can be used before or after the occurrence of an immune reaction.

- In one embodiment, this invention provides specific FVIII-lipid complexes.
- 20 The protein-lipid complexes may form novel lipidic structures as well as structures such as liposomes, cochleate, micelles and non-bilayer structures to reduce the immune response and antigenicity. The method involves developing specific FVIII-lipid complex preferably stabilized by buffer conditions. Although not intending to be bound by any particular theory, it is believed that the reduction in antigenicity and
- 25 immunogenicity arise from the protein lipid complex and in molecular assemblies such as liposomes would involve carrier properties such as hydrophobic shielding and cellular (antigen presenting cells APCs) uptake of particulate matters. These complexes have clotting activity in the presence of antibodies and exhibited reduced antigenicity as measured by their ability to bind to monoclonal antibodies in an ELISA assay. The
- 30 complexes also showed reduced immune response in animal models. The present invention is useful not only to reduce the immune response development in previously untreated patients with FVIII but also retain FVIII clotting activity in previously treated patients who have developed antibodies.

Accordingly, in one embodiment, the present invention provides a method for reducing the immune response against FVIII. By the use of the compositions disclosed herein, immunogenicity is reduced while the clotting activity is maintained. It is considered that the reduction in immunogenicity is accomplished by complexing of the phospholipids with the C2 and A2 domains. It is considered that this decreases immunogenicity by (1) reducing aggregate formation, (2) decreasing the frequency of administration (the complexes alter the clearance mechanism thus by providing longer circulation time) and/or (3) shielding and altering the conformation of the epitope region and/or (4) carrier properties that include preferential cellular uptake. The improved pharmaceutical properties of the complex such as stability, altered clearance mechanism to increase circulation time and reduced antigenicity and immunogenicity is an unexpected observation.

The following examples are presented to further describe the invention and not intended to be restrictive in any way.

EXAMPLE 1

This example demonstrates the preparation of protein-lipid complexes according to present invention. To illustrate this embodiment, FVIII was used. The FVIII-O-phospho-L-serine (OPLS) complex was formed by mixing 20ug of the protein with 5 and 20mM of the OPLS in 25mM TRIS, 300mM NaCl and 5mM CaCl₂.

EXAMPLE 2

This example describes the stability of the protein-lipid complexes of the present invention (Example. 1). For free FVIII the unfolding of the protein results in the aggregation of the protein and this in turn leads to the irreversibility of unfolding. The aggregation is initiated by small conformational changes in C2 domain. The unfolding/refolding studies were carried out with free FVIII and FVIII complexed to PS as described in Example 1 to determine the stability of the formulation containing protein and O-Phospho-L-Serine that is believed to bind to the C2 domain of FVIII.

Circular Dichroism (CD), fluorescence anisotropy, size exclusion chromatography (SEC), domain specific antibody binding and clotting activity studies were carried out to investigate the temperature dependent physical and functional changes of recombinant human FVIII (rFVIII). For determining the folding and

unfolding, CD spectral studies were carried out. CD spectra were acquired on a JASCO-715 spectropolarimeter calibrated with d10 camphor sulfonic acid. Samples were scanned in the range of 255 to 208 nm for secondary structure analysis, and typically, the protein concentration used was 20-22 $\mu\text{g/ml}$. CD spectra of the protein were corrected by subtracting the spectrum of the buffer baseline. Multiple scans were acquired and averaged to improve signal quality. Melting of the protein was followed over the temperature range of 20°C-80°C with a 2 min holding time at every 2.5°C. The temperature scans were acquired with a Peltier 300 RTS unit and the profiles were generated using the software provided by the manufacturer. Heating rate dependence of the unfolding profiles indicated that the thermal denaturation of the protein was at least in part under kinetic control (Figure. 1). A folding model was proposed to explain the aggregation kinetics of Factor VIII. Based on this model, unfolding of Factor VIII was interpreted in terms of the simple two-state kinetic model, Aggregated (A) \rightarrow Native (N) where k is a first-order kinetic constant that changes with temperature, as given by the Arrhenius equation. The activation energy associated with the above transition was calculated to be ~ 127.98 Kcal/Mole (~ 534.97 KJ/Mole). Antibody binding studies indicated that conformational changes in the lipid-binding region (2303-2332) of the C2 domain may at least in part be responsible for the initiation of aggregation (Figure. 2). Analysis of the SEC profile of FVIII in the presence and in the absence of OPLS clearly showed that the monomeric population is significantly higher than that of aggregated protein in the presence of PS, possibly due to the interference of OPLS in the aggregation kinetics of Factor VIII (Figure. 3). The data indicates that the complex improves the stability of FVIII and may help to reduce the immunogenicity by reducing the aggregates.

In a further illustration of this embodiment, O-phospho-L-serine was complexed with the protein (likely shielding the C2 domain). 20 $\mu\text{g/ml}$ of the protein was mixed with 5 and 20 mM of phospho-L-serine in different buffers including 25 mM TRIS, 300 mM NaCl and 5 mM CaCl_2 . The binding to the monoclonal antibodies was studied by sandwich ELISA. As shown in Figure 4, the recovery of native like structures were not possible for excipient free protein due to aggregation whereas presence of O-Phospho-L-Serine resulted in substantial recovery of native structure.

EXAMPLE 3

This example demonstrates that the protein-lipid composition of the present invention (Example. 1) reduces the immunogenicity against the protein in Sprague-Dawley rats. To illustrate this embodiment, OPLS-Factor VIII complex was administered to Sprague-Dawley rats. This rat model has been shown to be suitable to study antibody development to FVIII. The antibody titer measured by ELISA for free FVIII and FVIII-PS complex. Two weeks after the administration, the analysis of antibody titer for Factor VIII –OPLS complex was found to be non- immunotoxic. The antibody titers for free FVIII is 563.72 ± 916.15 and no detectable antibody titers was observed for FVIII-OPLS complex.

EXAMPLE 4

This example demonstrates that phosphoserine is needed in the simple complexes for the reduction in immunogenicity. To illustrate this example, simple complexes were prepared with phosphoserine, phosphocholine or phosphatidic acid. Immunogenicity was tested by subcutaneous administration of the complexes in Hemophilia A mice. The total antibody titers (as measured by ELISA) or specific inhibitory titers (Bethesda Units) titers are shown in Figures 5A and 5B. The effect was statistically significant for phosphoserine but not for phosphocholine or phosphatidic acid.

EXAMPLE 5

This example demonstrates that the composition of the present invention can be made as small unilamellar vesicles. 0.3mg/ml of DMPC and 0.15mg/ml of bPS dissolved in a round bottomed flask and the solvent was evaporated to form a thin film. The film was then hydrated to form MLV's and the MLVs were extruded through 200nm polycarbonate filters to form SUV's in the size range of 160 nm. The immune response of this formulation (containing Factor VIII) is described in Example 5.

In all the above mentioned studies, experiments performed using PS free conditions and the concentration of the protein associated with the lipid and lipidic structures are low indicating that the presence of specific lipid-FVIII complex in all these lipidic formulations. Further, the monoclonal antibody specific for C2 domain did not bind to FVIII in the presence of Serine clearly indicates the presence of complex in lipid structures studied.

EXAMPLE 6

This example demonstrates the general methodology of the ELISA assay to investigate the antigenicity and the participation of particular epitope region of the protein in forming the complex. The antibody binding of the protein-lipid complexes was investigated by antibody capture ELISA and sandwich ELISA. For Sandwich ELISA, 96 well plates (Nunc-Maxisorb) were coated with an anti-C2 domain antibody (ESH4) by incubating 50 μ l/well solution of the antibody at a concentration of 5 μ g/ml in carbonate buffer (0.2 M, pH 9.4) overnight at 4 °C. The plate was then washed 10 times with 100 μ l of Phosphate buffer containing 0.05% Tween 20 (PBT consisting of 10mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.14 mM NaCl, 2.7mM KCl, and 0.02% NaN₃). The remaining nonspecific protein binding sites on the plastic's adsorptive surface were blocked by incubating 200 μ l of blocking buffer consisting of 1% bovine serum albumin in phosphate buffer (PB consisting of 10mM Na₂HPO₄, 1.8 mM KH₂PO₄, 0.14 mM NaCl, and 2.7mM KCl) for 2 hours at room temperature. The plates were washed 10 times with PBT and 50 μ l of 100 ng/ml of rFVIII or rFVIII/OPLS (Examples 1 and 3) or Liposome associated rFVIII (Examples 5) in blocking buffer was added and incubated at 37°C for 1 hour. The plates were washed 10 times with PBT and incubated with 50 μ l of biotinylated ESH8 - another anti-C2 antibody, at 1 μ g/ml concentration and 50 μ l of a 1:1000 dilution of avidin-alkaline phosphatase conjugate, both in blocking buffer at room temperature for 1 hour. The plates were washed 10 times with PBT and 100 μ l of 1 mg/ml p-nitrophenyl phosphate solution in diethanolamine buffer (consisting of 1M diethanolamine, 0.5 mM MgCl₂ and 0.02% NaN₃). The plates were incubated at room temperature for 30 minutes and the reaction was quenched by adding 100 μ l of 3 N NaOH. Absorbance was read by a plate reader at 405 nm. The ELISA studies indicated that less C2 domain specific antibodies, ESH8 or ESH4 bound to the protein in the presence of PS (OPLS or liposomes Figure. 6). The results showed that the binding of these monoclonal antibodies were inhibited by lipid suggesting that epitope regions are shielded in the protein-lipid complex. Further, the specific complex FVIII-PS is responsible for the the epitope shielding is further confirmed by the control experiment in which the antigenicity was observed only with DMPC/PS mixture but not with DMPC alone (Figure. 7).

EXAMPLE 7

This example demonstrates that the protein-lipid composition of the present invention (Example. 5) reduces the immunogenicity against the protein in animal models, Sprague-Dawley rats and Factor VIII knock out mice, Hemophilia A model.

5 The antibody titres evaluated in Sprague-Dawley rats at 4th and 6th weeks post administration of the protein-liposomes complex was found to be lower for FVIII-PS complex compared to free FVIII (Figure. 8A).

10 In another illustration of this embodiment, the effect of protein-lipid complexes of the present invention on immunogenicity was tested in a Factor VIII knock out mice model. The phenotype of the mice is severe hemophilia. (exon 16 knock-out by targeted disruption using a neo cassette). The protein-liposome complex (DMPC + bPS) was administered subcutaneously and the antibody titres were measured using ELISA assay. As is clear from the Figure 8B, the antibody concentrations were lower for the liposome bound protein after 6 weeks of administration. The data clearly
15 demonstrates that liposome bound Factor VIII elicits less antibodies compared to free factor VIII. Although not intending to be bound by any particular theory, this observations may partly be due to altered (1) conformation and aggregation kinetics of free FVIII, (2) decoy effect of particulate material and processing by immune system and (3) clearance mechanism.

20

EXAMPLE 8

The following examples illustrate the formation of protein-lipid complexes in micelles, non-bilayered structures, cochleate structures and in novel non-liposomal lipid particles. This example describes the formation of micelles by the compositions
25 described herein. The protein solution was mixed with a shorter acyl chain lipids (Dihexanoyl phosphatidyl Serine (below and above 0.3mM) at lower and higher concentration (below and above critical micellar concentrations) and the resulting micellar particles were characterized. The structure of the micelles were characterized by light scattering, circular dichroism and fluorescence studies. The functional assays
30 such as activity and antibody binding were carried out.

EXAMPLE 9

This example describes the formation of cochleate structures by the

compositions described herein. 0.15mg/ml of bPS was dissolved in chloroform and the solvent was evaporated to form a thin lipid film. The film was then hydrated in several buffer system at pH 7.0 and the MLVs were either extruded or sonicated to form SUVs. The resulting SUVs were mixed with protein in buffer system containing 5mM CaCl_2 to form cochleate structure. The lipid structures were analyzed by light scattering, differential interference microscopy, negative stain electron microscopy and by fluorescence studies. These studies showed that the lipid structures formed by this procedure were cochleate in nature.

10 EXAMPLE 10

This example is another illustration of the preparation of protein-lipid complexes of the present invention. 0.15mg/ml of bPS and 0.3mg/ml of dioleoyl phosphatidylethanolamine (DOPE) was dissolved in chloroform and the solvent was evaporated to form a thin lipid film. The resulting lipid film was hydrated with phosphate buffered saline to form hexagonal phases. The non-bilayer structures were characterized by fluorescence studies.

EXAMPLE 11

This example is another illustration of the preparation of protein-lipid complexes of the present invention in a novel non-liposomal structures. 0.3mg/ml of DMPC, 0.15 mg/ml of bPS were dissolved in chloroform and the solvent was evaporated to form a thin lipid film in a round bottom flask. The lipid was hydrated using a buffer system containing FVIII, 25mM TRIS, 300mM NaCl and 5mM CaCl_2 and the solution was gently swirled either at room temperature at 37°C. The film was then hydrated in appropriate buffer (25mM TRIS, 300mM NaCl and 5mM CaCl_2), with gentle swirling. The MLV's thus formed were subjected to dextran centrifugation gradient to separate the free protein from protein associated with MLV's. 0.5ml of the lipid associated protein was mixed with 1ml of 20% w/v of dextran and a 3ml of 10% w/v dextran was layered over the above solution. Then 0.5ml of buffer layered on top. The gradient was centrifuged for 35 min at 45K RPM using Beckman SW50.1 rotor. The results of the centrifugation study is shown in Figure 9. As is clear from the figure, there are some lipidic fractions that could not be floated and are denoted as fraction 3 in the figure. This fraction was observed at the interface of 14% and 10% dextran. The

fact that this fraction could not be floated indicates that this lipidic fraction does not have enough buoyancy or encapsulated water. Conventional liposomes generally float to the top of the gradient because of their entrapped water. Therefore, the fraction that does not float may be a non-liposomal protein containing lipidic particles. The fraction
5 was collected and tested for lipid content by mass spectrometry and for protein content by activity. The mass spectroscopy studies showed that this fraction contained lipids including high PC content suggesting that it is not just PS-Ca⁺ complex. The activity assay showed approximately 40% of the initial protein was encapsulated in this fraction.

10 There are several possible explanations for the dense fraction 3 which has no or little water content. This fraction may represent: (1) very small unilamellar vesicles with less encapsulated water volume. In order to determine if smaller vesicles (less than 200nm) could be floated under identical conditions, pre sized liposomes were prepared by extruding through polycarbonate filters. The extrusion was repeated 3
15 times and the size of the particles was determined to be around 160nm. The resulting SUVs were mixed with FVIII and were subjected to Dextran centrifugation gradient. This control study showed that these SUV's did not show fraction 3 band indicating that the observation of such bands is not due to the formation of small liposomes. Further, this experiment was performed under identical buffer and experimental conditions to
20 rule out any artifacts in the dextran gradient. (2) The second possibility is that fraction 3 may represent the formation of cochleate structures, which have less water content [26]. However, the formation of cochleate structures needs a very high PS content (>50%). In the formation of fraction 3 band, the PS content used was around 30% and under these conditions the formation of cochleate structures has not been shown. (3) The
25 formation of collapsed Ca(PS)₂ complex that has a dehydrated structure [27] may not float and can form a dense band. The formation of such collapsed structure requires very high PS content (>50%) but in the absence of Na⁺. However, in the present example, the composition contains low PS content and a very high concentration of Na⁺ i.e., 30% PS and 300mM NaCl is used and therefore, the possibility of Ca(PS)₂
30 formation is ruled out. (4) The PS and calcium system has been shown to promote vesicle fusion. However, the fusion of vesicles by divalent cations such as Ca²⁺ is inhibited by the presence of Na⁺ as it competes with calcium for the lipid binding site. The estimated amount of Calcium bound per PS in this PC/PS ratio of 7:3 and in the

presence of 300mM NaCl and 5mM CaCl_2 , is between 0.22 (500mM NaCl) to 0.35 (100mM NaCl) [28, 29]. This estimated bound calcium per PS is less than the critical ratio of 0.35 to 0.39 required for fusion in a small unilamellar PS/PC vesicle system. Thus, the fusion of vesicles does not appear to be represent the dense band. This is
5 because the larger PC fraction (>50%) may result in less PS-divalent cation complex and its ability to cluster into large domains to induce fusion)[28, 29]. Based on these arguments, it appears that the dense band may be due to the formation of novel, non-liposomal lipid particles. This band is non-liposomal because of less encapsulated water volume. In order to understand the structure of this novel lipidic structure, negative
10 stain electron microscopy, differential contrast interference optical microscopy, light scattering, circular dichroism and fluorescence measurements (data not shown) were performed.

Centrifugation studies carried out under several conditions indicate that buffer conditions, bulk protein concentration, use of alcohol (such as 10% ethanol) and
15 protein-lipid ratio can be varied to obtain the desired yield of the complexes. Such variations in these parameters are considered to be within the purview of one skilled in the art. In general, it was observed that higher protein concentration resulted in more intense dense fraction as confirmed by visual inspection.

20 EXAMPLE 12

This example demonstrates that the protein-lipid compositions of the present invention retain their biological activity. To illustrate this embodiment, the effect of protein-lipid complex (OLPS, DCPS, and DCPC) comprising Factor VIII on clotting was tested. rFVIII clotting activity was determined by one-stage activated partial
25 thromboplastin time (APTT) assay using micronized silica as activator and FVIII deficient plasma as the substrate. The APTT assay was performed using a COAG-A-MATE model coagulation analyzer (Organon Teknika Corporation, Durham, NC). Briefly, rFVIII was added to FVIII deficient plasma and the clotting time was monitored. The activity of the rFVIII was then obtained from calibration curve
30 constructed using the clotting times determined from various dilutions of a lyophilized reference concentrate of known activity. The concentration of the protein was determined independently using Bicinchoninic acid (BCA) assay and compared with activity. For example, all the 20-22 $\mu\text{g}/\text{ml}$ of the protein corresponds to specific activity

of 87 - 95.6 IU. The stock solution used to prepare the samples had a specific activity of 2174 IU/0.5 mg/ml.

EXAMPLE 13

5 This example demonstrates reduced antigenicity of proteins in the presence of PS containing phospholipids. To illustrate this embodiment, the effect of dicaproyl phopatidylserine (DCPS) on the binding of rFVIII to ESH4 antibody was tested. The FVIII- (DCPS) complex was formed by mixing 2ug of the protein with 0.5, 2 and 5 mM of the DCPS in 25mM TRIS, 300mM NaCl and 5mM CaCl₂. The antigenicity of
10 the complex was investigated using ELISA. As shown in Figure 10, DCPS inhibited the binding of rFVIII to ESH4 antibody at the concentrations tested i.e., 0.5, 2 and 5.0 uM. These concentrations are above and below the CMC of this phospholipid.

EXAMPLE 14

15 This example describes the effect of PS containing phospholipids on the immunogenicity of proteins. The FVIII- (DCPS) complex was formed by mixing 2ug of the protein with 5 mM of DCPS in 25mM TRIS, 300mM NaCl and 1mM CaCl₂. This composition forms micellar structure and the immunogenicity of the complex was investigated in Hemophilia A mice. The total antibody titers for free FVIII is 13,167
20 (SD = 7909, n=16) and for FVIII-DCPS complex is 3506 (SD=1150.7, n=12).

EXAMPLE 15

 The FVIII- Dicaproyl Phosphatidyl Choline (DCPC) complex was formed by mixing 2ug of the protein with 20mM of DCPC in 25mM TRIS, 300mM NaCl and
25 1mM CaCl₂. This composition forms micellar structure and the immunogenicity of the complex was investigated in Hemophilia A mice. The total antibody titers for free FVIII is 13,167 (SD = 7909, n=16) and for FVIII-DCPC complex is 1293 (SD= 946.57, n=12).

EXAMPLE 16

30 This example demonstrates that the composition of the present invention can be made as liposomal vesicles. 11.25μmol of DMPC and 4.83 μmol of BPS dissolved in choloroform were taken in a round-bottomed flask and the solvent was evaporated

using a rota-evaporator to form a thin film on the walls of the flask. The film was then hydrated in 3ml Tris buffer (containing 300mM NaCl, 25mM Tris, 5mM CaCl₂, pH=7.0) to form MLV's and the MLVs were extruded through either 80nm or 200nm or 400 nm polycarbonate filters to form SUV's in the size range of 80 nm to 400 nm. Lipid recovery was estimated by determination of phosphorous content by the method of Bartlett. The protein was associated with liposomes by incubating at 37°C for 30 minutes. The liposomes were then be used in immunizations as follows. Immunization of FVIII knockout mice consisted of four subcutaneous (s.c.) injections of rFVIII or rFVIII-liposomes (2 µg in 100 µl of Tris buffer) at weekly intervals. The molar ratio between the protein and lipid was maintained at 1:10,000.

In another sample, 11.25 µmol of DMPC dissolved in choloroform was taken in a round-bottomed flask and the solvent was evaporated using a rota-evaporator to form a thin film on the walls of the flask. The film was then hydrated with 3ml Tris buffer (containing 300mM NaCl, 25mM Tris, 5mM CaCl₂, pH=7.0) to form MLV's and the MLVs were extruded through either 80nm or 200nm or 400 nm polycarbonate filters to form SUV's in the size range of 80 nm to 400 nm. Lipid recovery was estimated by determination of phosphorous content by the method of Bartlett. The protein was associated with liposomes by incubating at 37°C for 30 minutes. Immunization of FVIII knockout mice consisted of four subcutaneous (s.c.) injections of rFVIII or rFVIII-liposomes (2 µg in 100 µl of Tris buffer) at weekly intervals. The molar ratio between the protein and lipid was maintained at 1:10,000. DSPC having a high phase transition temperature (solid state) was used a negative control.

In another sample, 7.5 µmol of DMPC and 3.22 µmol of DOPA dissolved in choloroform were taken in a round-bottomed flask and the solvent was evaporated using a rota-evaporator to form a thin film on the walls of the flask. The film was then hydrated with 2ml Tris buffer (containing 300mM NaCl, 25mM Tris, 5mM CaCl₂, pH=7.0) to form MLV's and the MLVs were extruded through 200nm polycarbonate filters to form SUV's in the size range of ~200nm. Lipid recovery was estimated by determination of phosphorous content by the method of Bartlett. The protein was associated with liposomes by incubating at 37°C for 30 minutes. Immunization of FVIII knockout mice consisted of four subcutaneous (s.c.) injections of rFVIII or rFVIII-liposomes (2 µg in 100 µl of Tris buffer) at weekly intervals. The molar ratio between the protein and lipid was maintained at 1:10,000.

In another sample, 7.5 μ mol of DMPC and 3.22 μ mol of DOPG dissolved in chloroform were taken in a round-bottomed flask and the solvent was evaporated using a rota-evaporator to form a thin film on the walls of the flask. The film was then hydrated with 2ml Tris buffer (containing 300mM NaCl, 25mM Tris, 5mM CaCl₂, pH=7.0) to form MLV's and the MLVs were extruded through 200nm polycarbonate filters to form SUV's in the size range of ~200 nm. Lipid recovery was estimated by determination of phosphorous content by the method of Bartlett. The protein was associated with liposomes by incubating at 37°C for 30 minutes. Immunization of FVIII knockout mice consisted of four subcutaneous (s.c.) injections of rFVIII or rFVIII-liposomes (2 μ g in 100 μ l of Tris buffer) at weekly intervals. The molar ratio between the protein and lipid was maintained at 1:10,000. The immune response of this formulation is described in Table 1.

Table 1

Composition	Size* (nm)	Lamellar Phase State at 37°C	Total Anti-rFVIII Antibody Titers (Mean \pm S.E.M)	Anti-rFVIII Inhibitory Titers (BU/ml)(Mean \pm S.E.M)
1. rFVIII (Control)	-	-	13166.7 \pm 2042.2 (n=15)	689.7 \pm 78.2 (n=13)
2. DMPC/BPS	200	Fluid	5699.9 \pm 1254.7 (n=15)**	215.4 \pm 70.9 (n=14)**
3. DMPC	200	Fluid	6075.3 \pm 1114.4 (n=15)**	271.3 \pm 82.1 (n=14)**
4. DMPC/DOPA	200	Fluid	5640.4 \pm 1208.1 (n=15)**	185.4 \pm 53.6 (n=13)**
5. DMPC/DOPG	200	Fluid	5592.4 \pm 950.3 (n=15)**	299.2 \pm 87.9 (n=14)**
6. DMPC	400	Fluid	6403 \pm 1730 (n=12)**	245.0 \pm 72.1 (n=12)**
7. DMPC/BPS	400	Fluid	4030 \pm 495.8 (n=12)**	160.6 \pm 27.3 (n=12)**
8. DMPC	80	Fluid	9680.6 \pm 2585.9 (n=12)	261.5 \pm 53.2 (n=12)**
9. DMPC/BPS	80	Fluid	7056.6 \pm 806.9 (n=12)**	257.7 \pm 35.4 (n=12)**
10. DSPC	200	Gel	9823.7 \pm 1401.9 (n=12)	532.2 \pm 111.8 (n=12)

Mean total anti-rFVIII antibody titers and inhibitory titers following immunization of FVIII-KO mice with rFVIII in the absence and presence of liposomes at the end of six weeks. *, indicates the pore size of polycarbonate membranes used to extrude liposomes, **p<0.05, statistical analysis was carried out as described under
 5 Experimental Procedures.

While this invention has been described through examples presented herein, routine modifications can be made to the invention without departing from the spirit of the invention. Such modifications are intended to be within the scope of the claims.

10

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Claims:

1. A method for reducing the immunogenicity of a therapeutic agent selected from the group consisting of peptide, polypeptide and protein comprising the steps of
5 forming a complex of the therapeutic agent with one or more binding agent selected from the group consisting of a serine compound, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidic acid (PA), and phospholipids comprising PC, PE or PA, wherein the immunogenicity of the therapeutic agent upon administration to an individual is reduced when complexed with the binding agent.
10
2. The method of claim 1, wherein the serine compound is selected from the group consisting of phosphoserine, phosphatidylserine (PS) and phospholipids containing PS.
3. The method of claim 1, wherein the complex comprises one or more structures
15 selected from the group consisting of simple complexes, liposomes, micelles, cochleates and non-liposomal lipidic structures, wherein the non-liposomal lipidic structures run at the interface of 10% and 14% dextran gradient.
4. The method of claim 3, wherein the binding agent is phosphoserine and the
20 complex formed is simple complex.
5. The method of claim 4, wherein the phosphoserine is O-phospho-L-serine.
6. The method of claim 1, wherein the phospholipids are acylated or diacylated.
25
7. The method of claim 6, wherein the acyl chain length is between 1 and 18 carbon atoms.
8. The method of claim 7, wherein the acyl chain length is between 1 and 4 carbon
30 atoms.
9. The method of claim 7, wherein the acyl chain length is between 5 and 12 carbon atoms.

10. The method of claim 7, wherein the acyl chain length is between 13 and 18 carbon atoms.
- 5 11. The method of claim 6, wherein the phospholipid is PS containing phospholipid.
12. The method of claim 11, wherein the phospholipid further comprises PC containing phospholipid and/or PE containing phospholipid.
- 10 13. The method of claim 12, wherein the ratio of PC containing phospholipids to PS containing phospholipids is 7:3.
14. The method of claim 6, wherein the acyl chains are selected from the group consisting of caproyl, oleoyl, octanoyl, myristoyl, palmitoyl, and steroyl.
- 15 15. The method of claim 1, wherein the therapeutic agent is Factor VIII.
16. The method of claim 1, wherein the non-lipid bilayers are formed by incubating the therapeutic agent with a serine containing phospholipid in the presence of about 100 nM of a sodium salt and about 5 nM of a calcium salt
- 20 17. Lipid-protein complexes which are formed by incubating a protein with a serine containing phospholipid in the presence of about 100 nM of a sodium salt and about 5 nM of a calcium salt and characterized in that the complexes migrate at the interface of 10% and 14% dextran gradient.
- 25 18. The lipid-protein complexes of claim 17 wherein the serine containing phospholipid is phosphatidyl serine.
- 30 19. The lipid-protein complexes of claim 18 further comprising phosphatidyl choline and wherein the ratio of phosphatidyl choline to phosphatidyl serine is 7:3.
20. The lipid-protein complex of claim 19 wherein the protein is Factor VIII.

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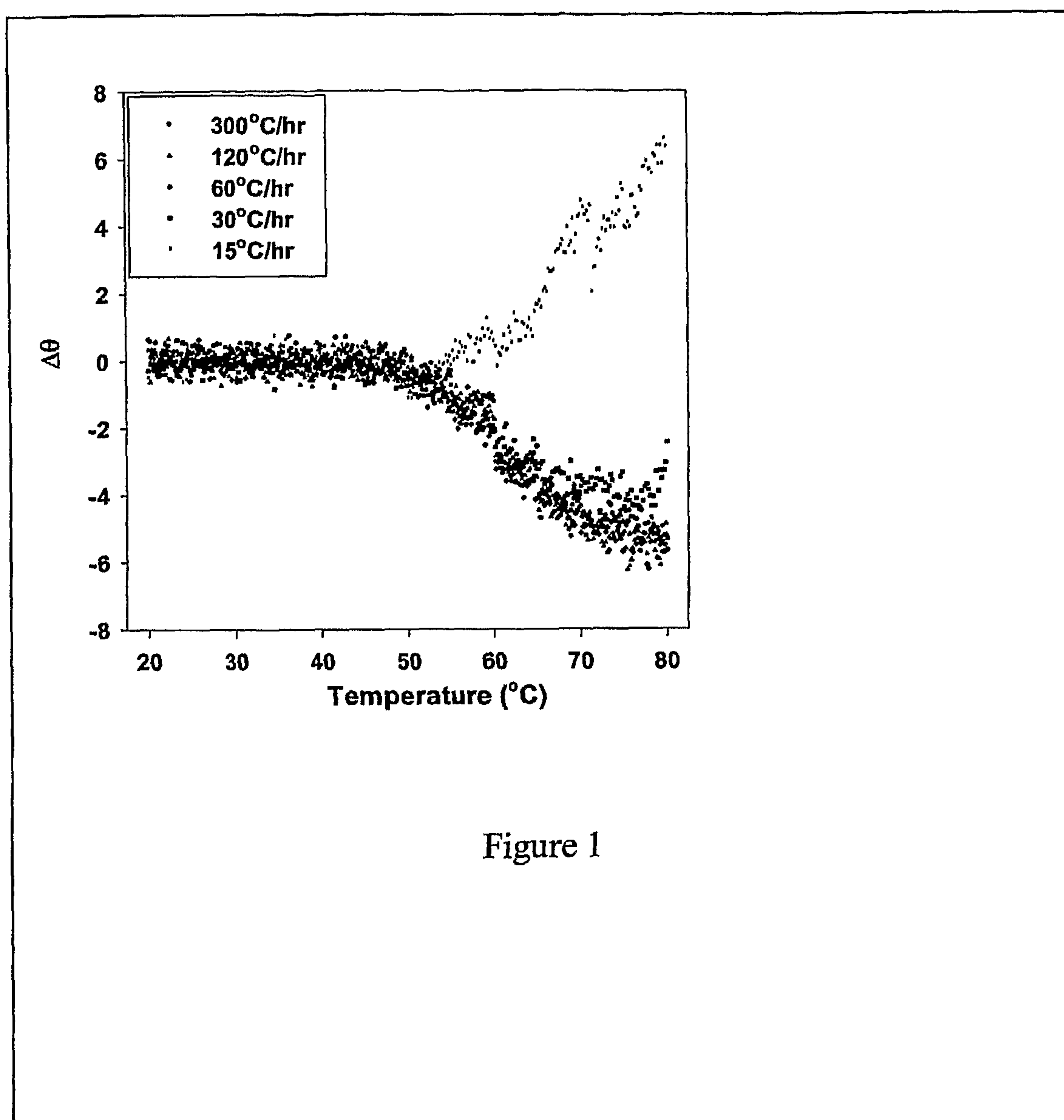
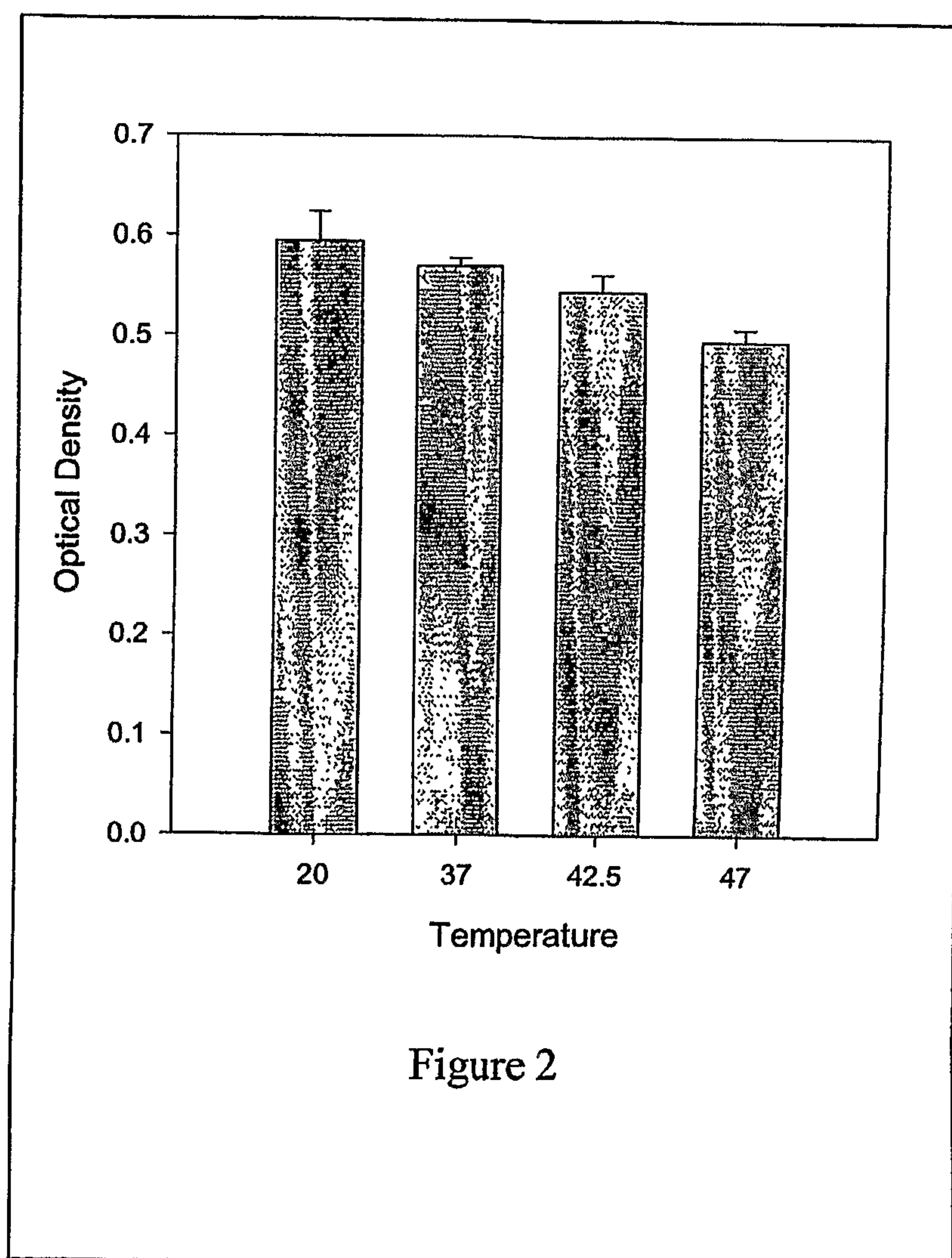


Figure 1

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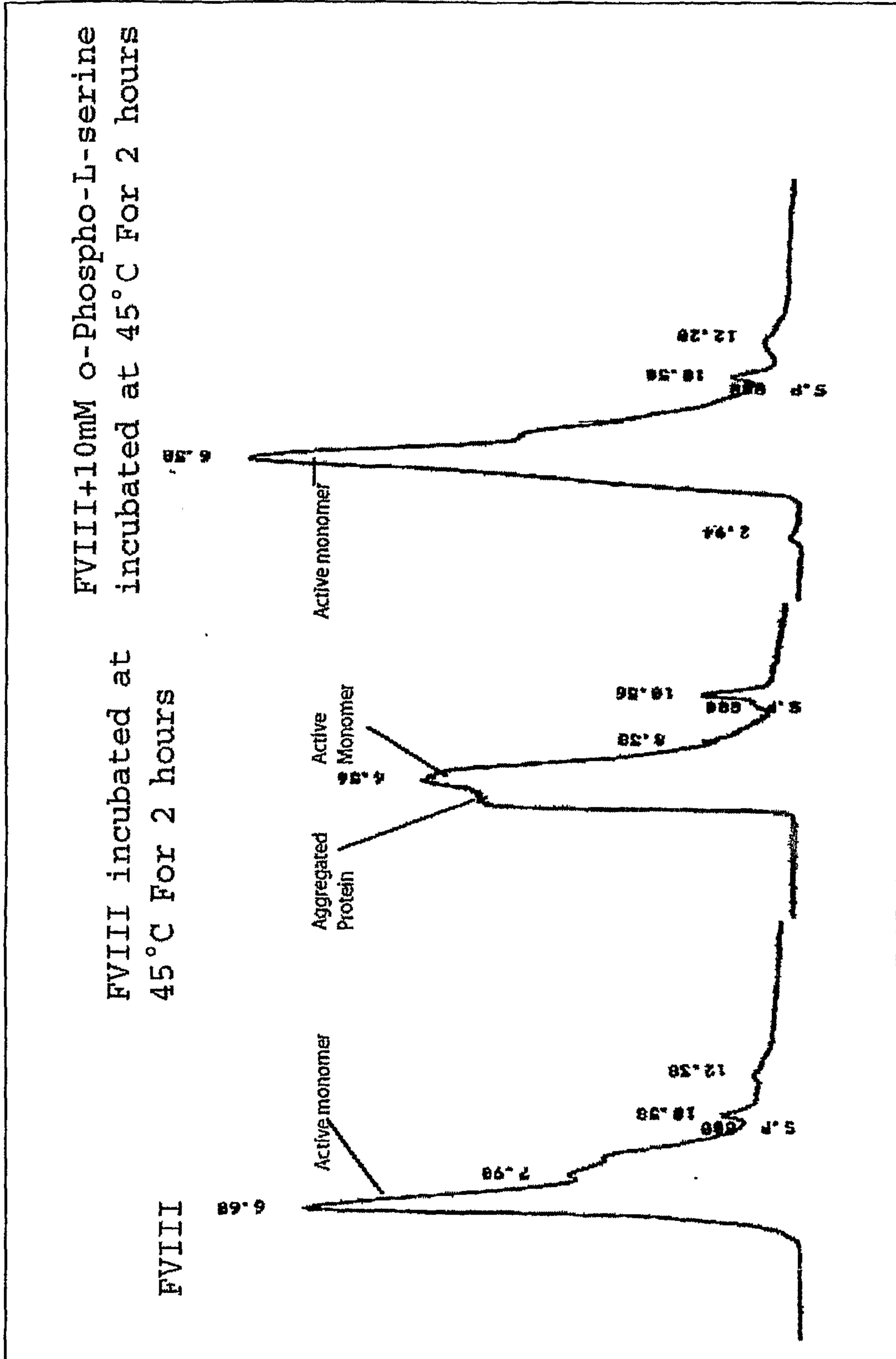


Figure 3

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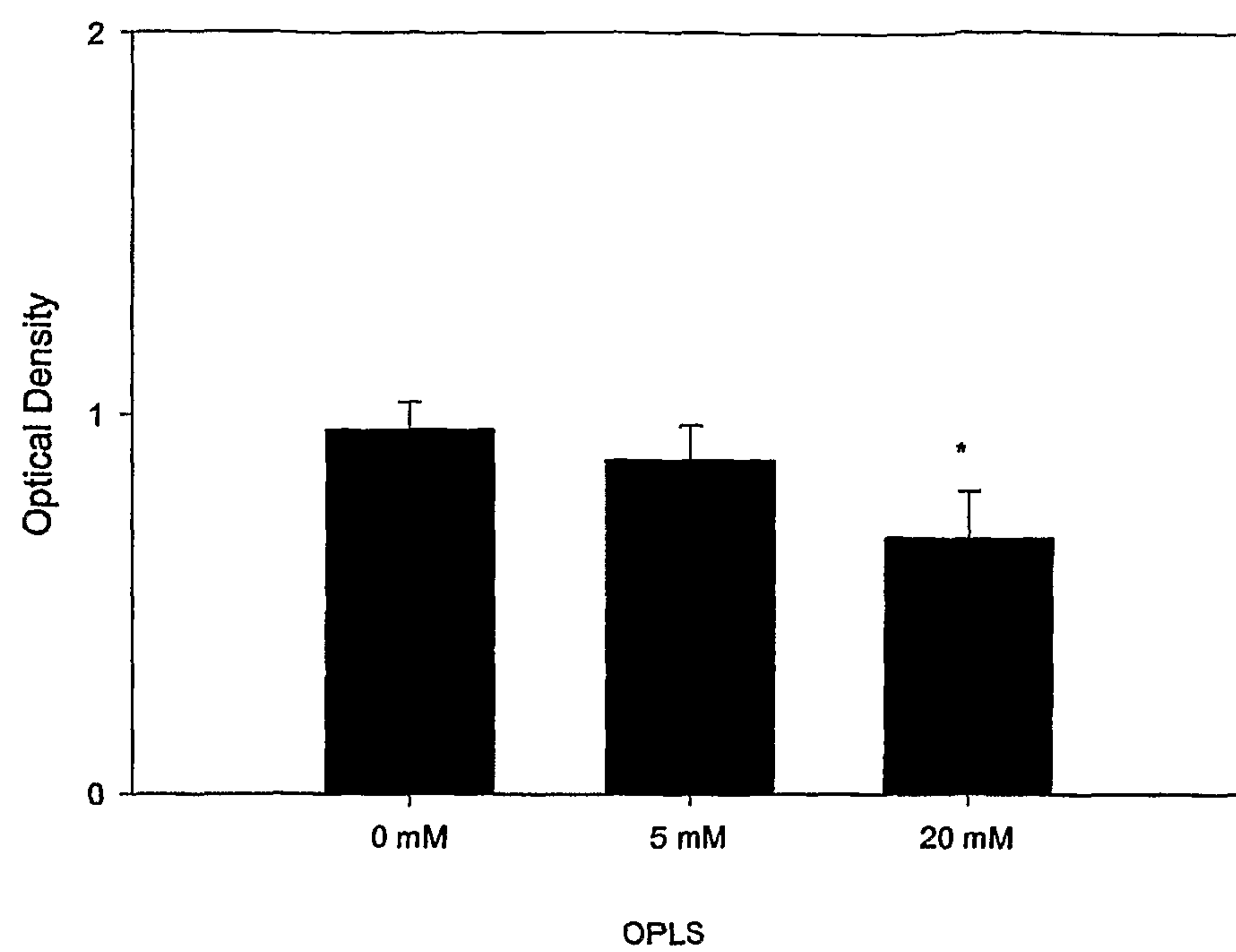


Figure 4

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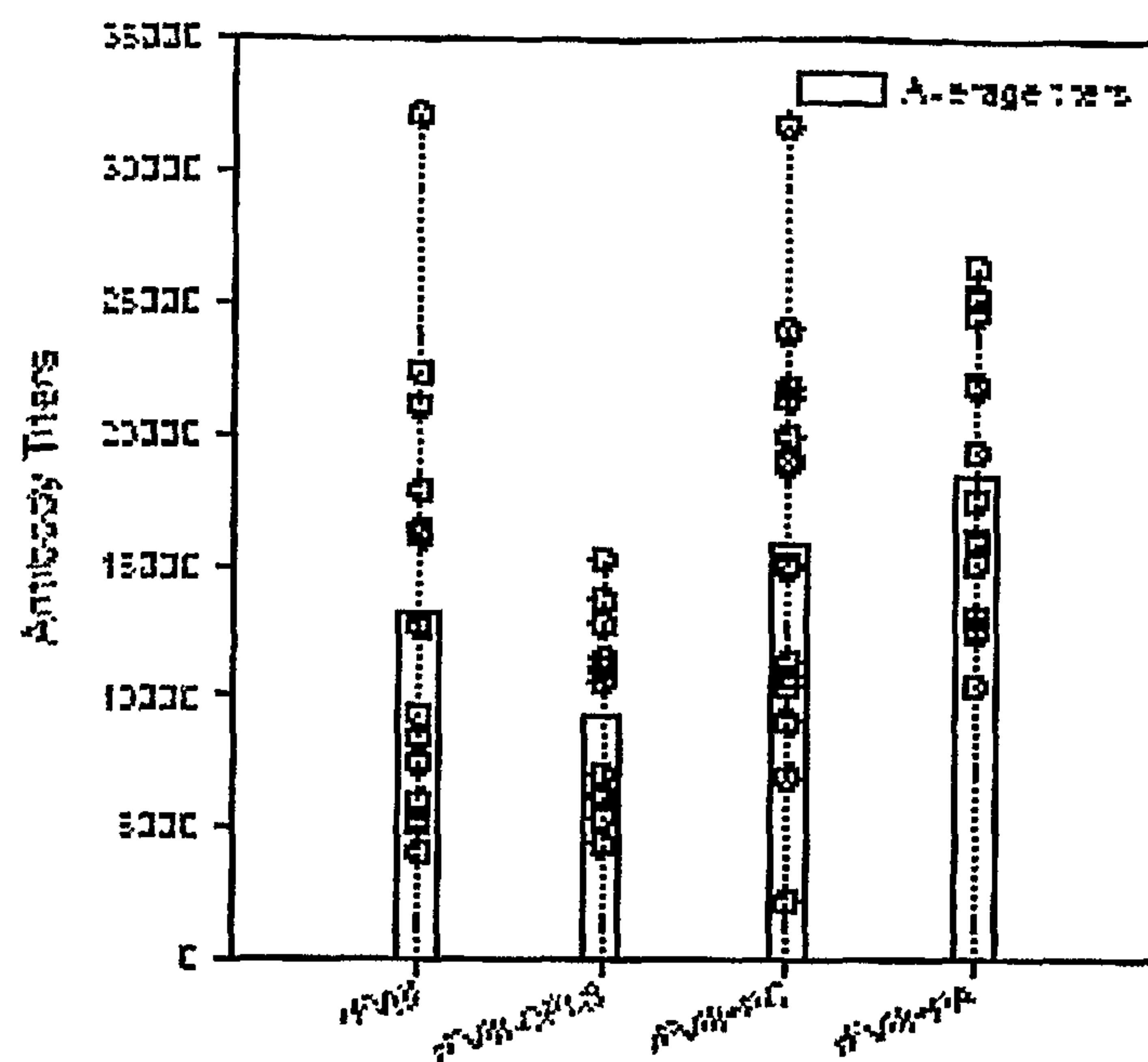


Figure 5A

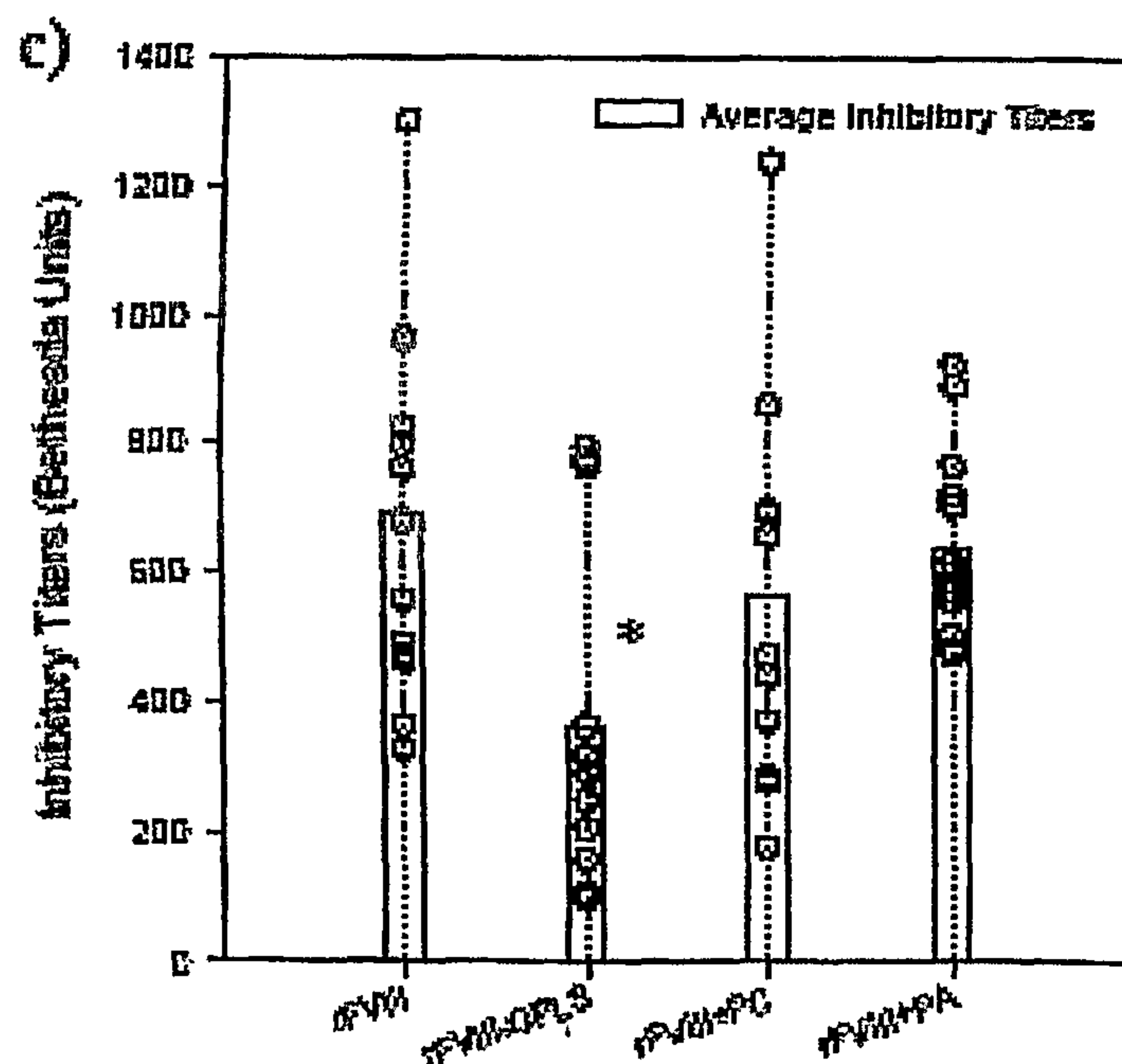
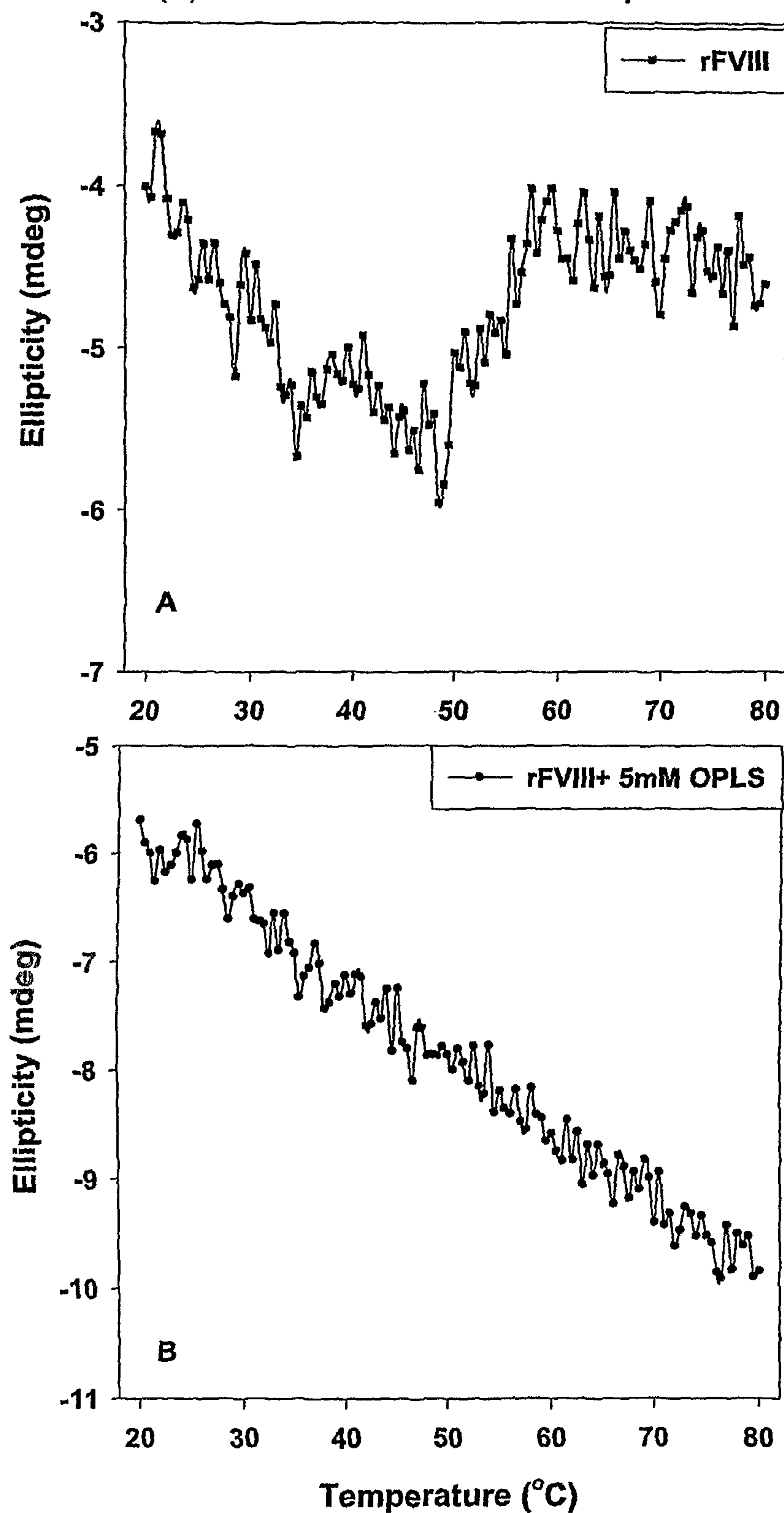


Figure 5B

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Secondary Structure Unfolding of rFVIII in the
Absence (A) and Presence of 5mM O-Phospho-L-Serine (OPLS)



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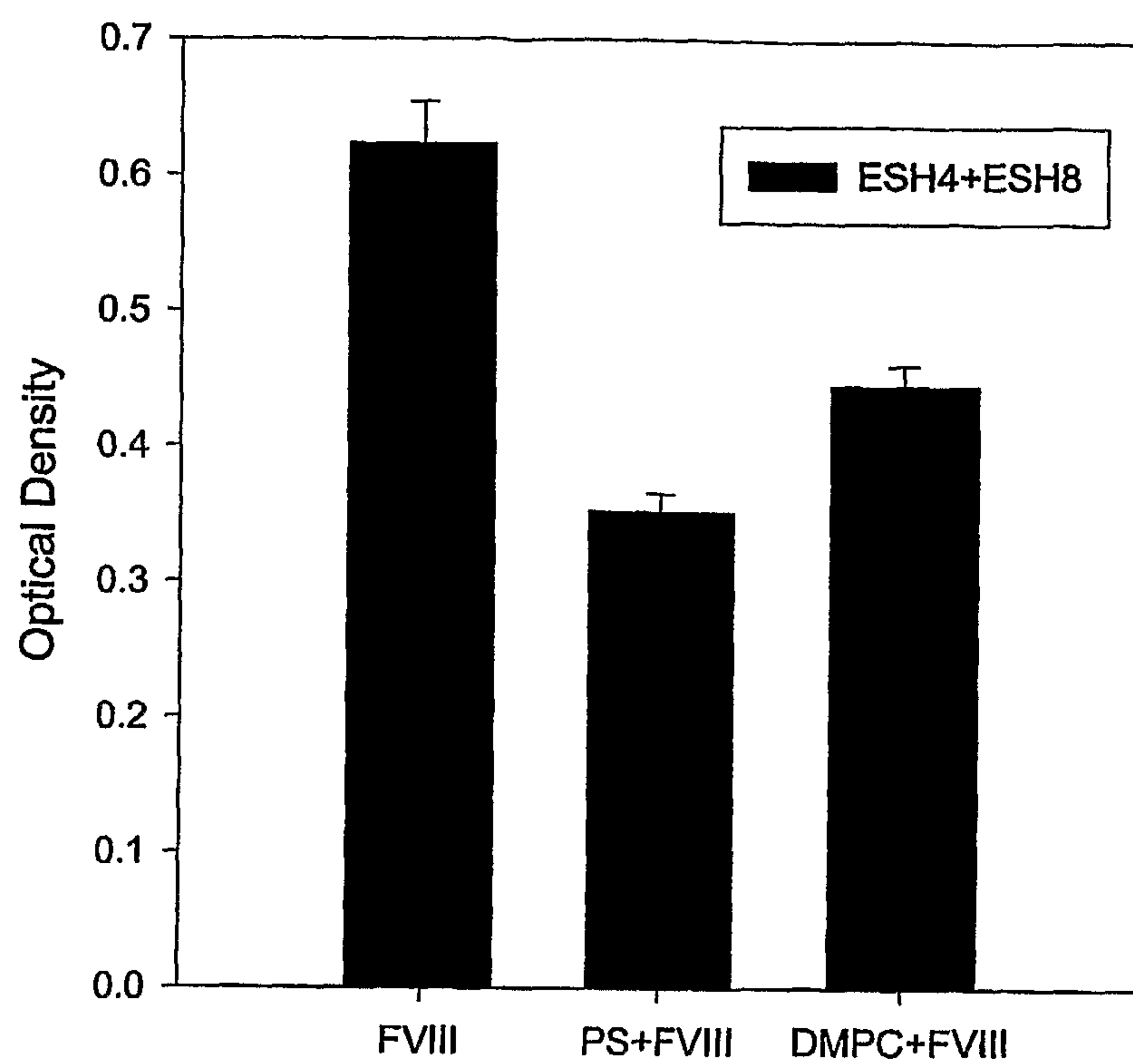


Figure 7

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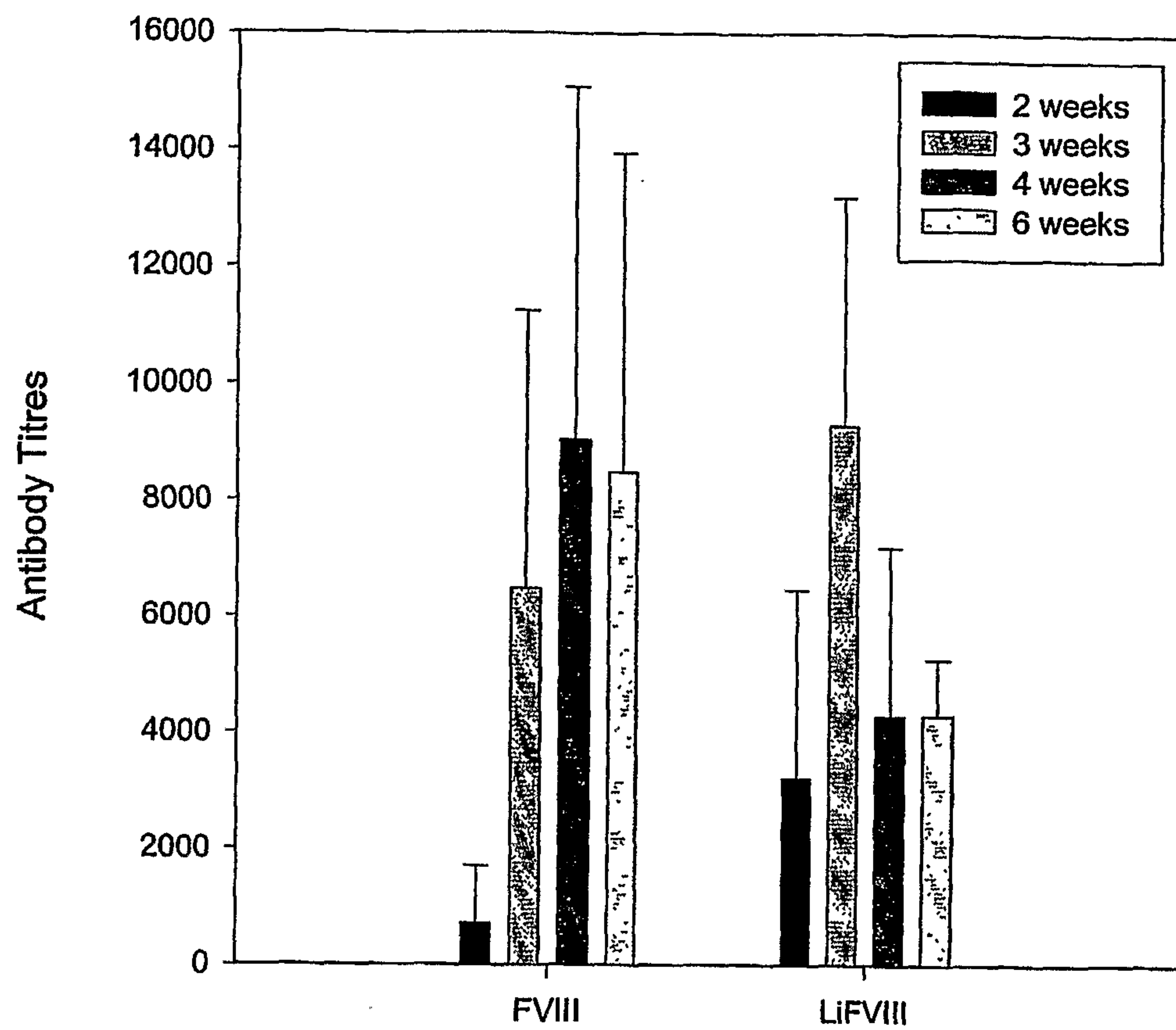
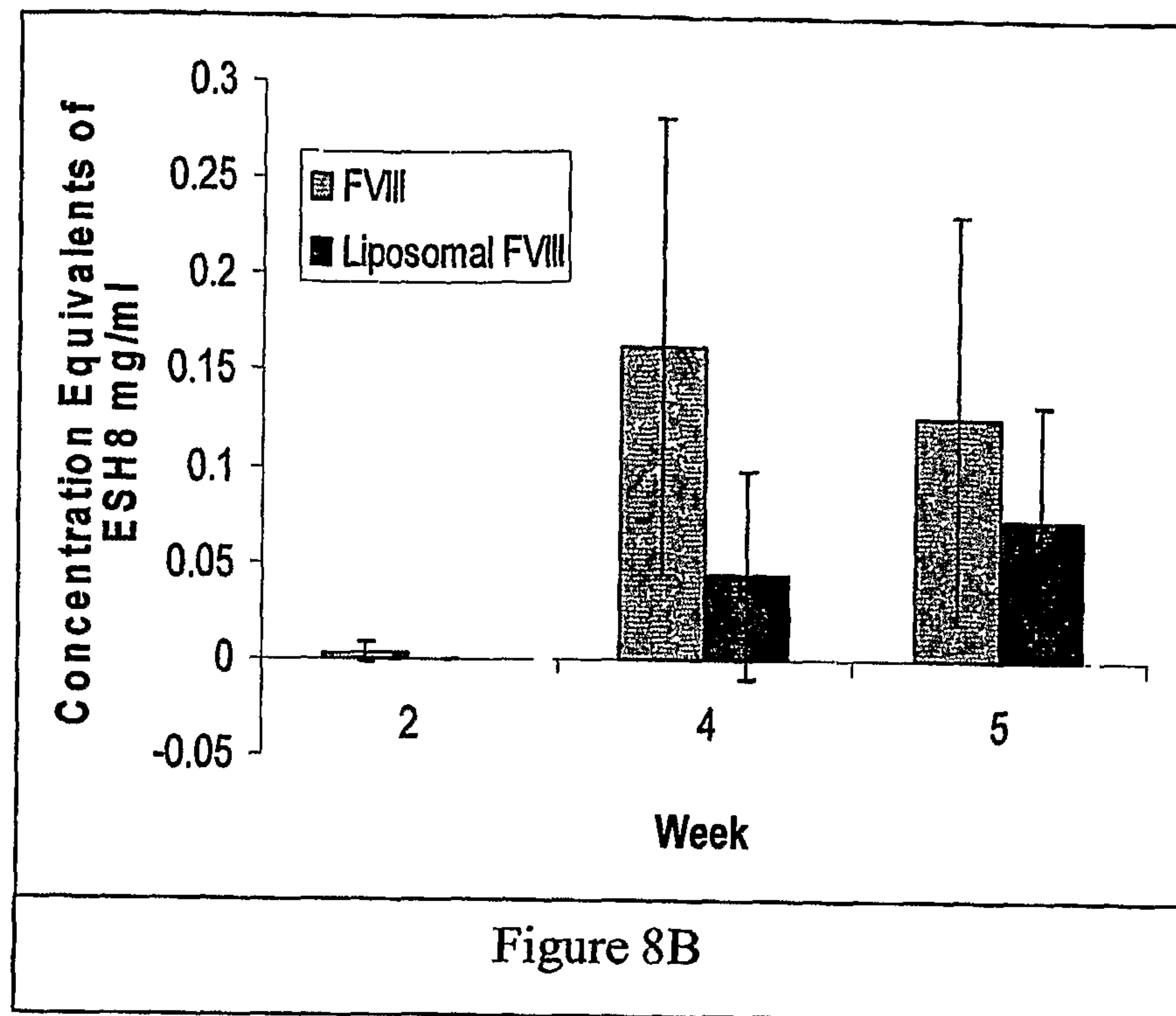


Figure 8A

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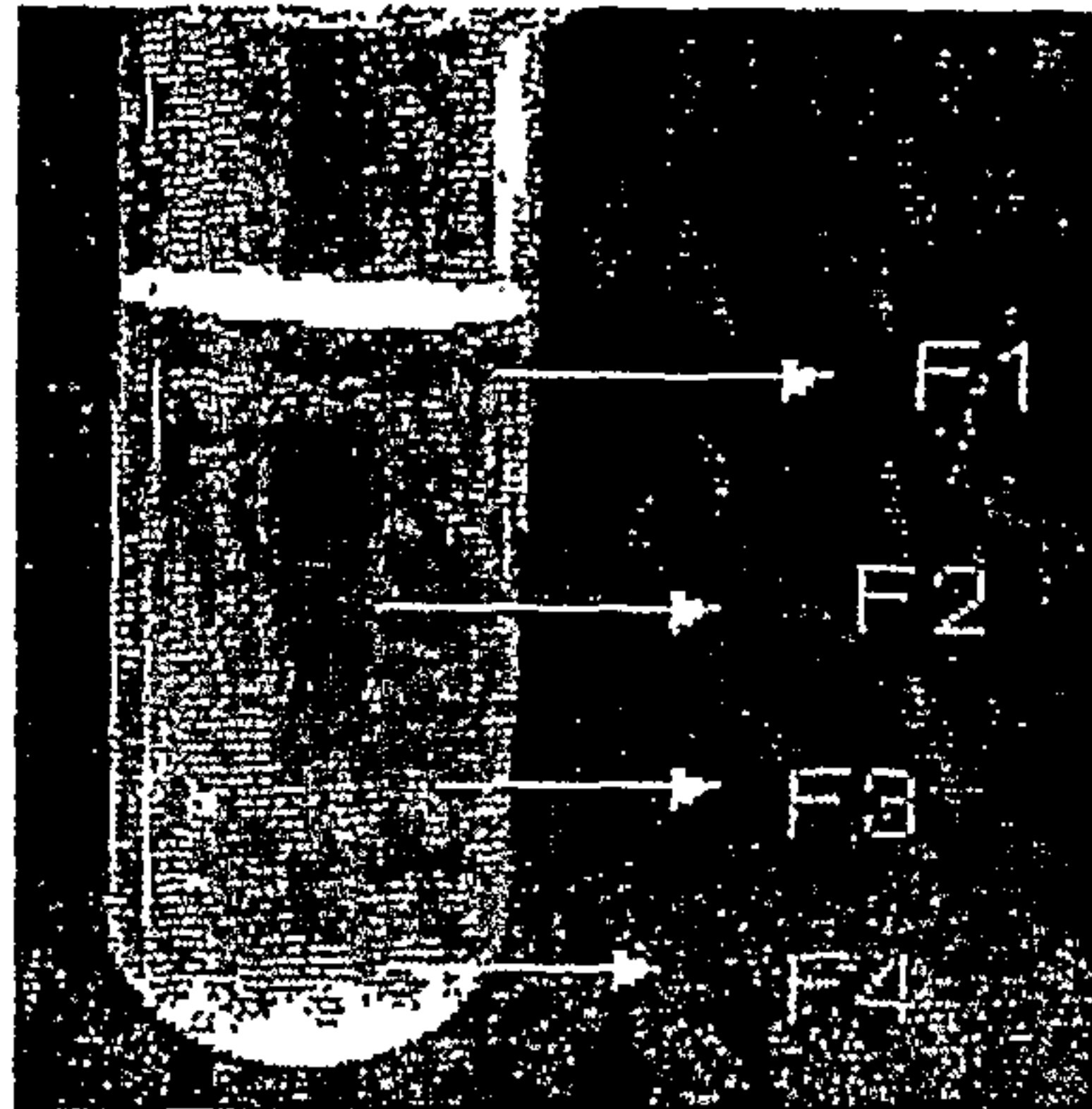


Figure 9

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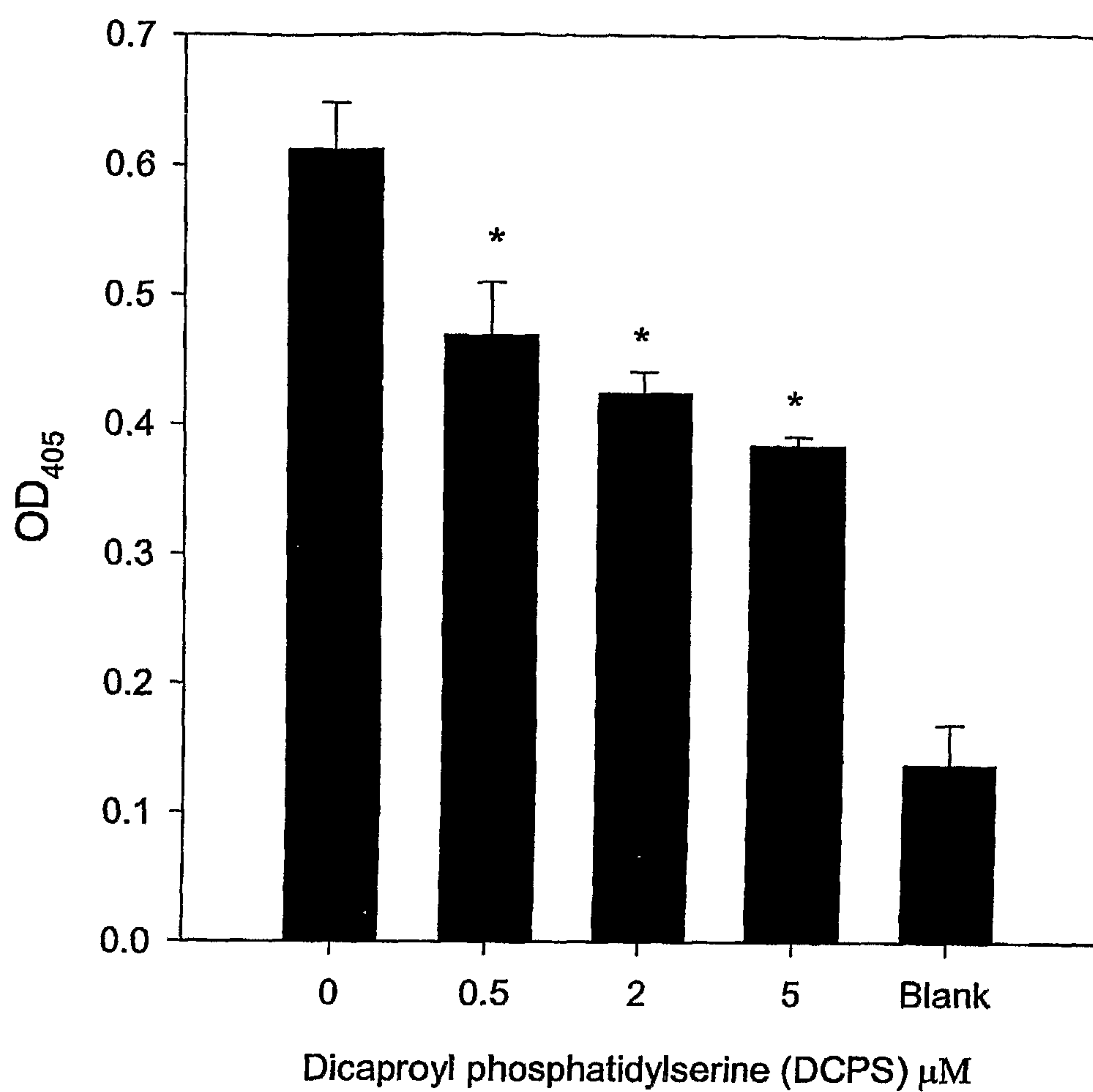


Figure 10