

Interaction of Dicaproyl Phosphatidylserine With Recombinant Factor VIII and Its Impact on Immunogenicity

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ABSTRACT

Replacement therapy with exogenous recombinant factor VIII (rFVIII) to control bleeding episodes results in the development of inhibitory antibodies in 15% to 30% of hemophilia A patients. The inhibitory antibodies are mainly directed against specific and universal immunodominant epitopes located in the C2 domain. Previously we have shown that complexation of O-phospho-L-serine (phosphatidylserine head group) with the phospholipid binding region of the C2 domain can lead to an overall reduction in the immunogenicity of rFVIII. Here, we have investigated the hypothesis that dicaproyl phosphatidylserine, a short-chain water-soluble phospholipid, can reduce the immunogenicity of rFVIII. Circular dichroism and fluorescence spectroscopy studies suggest that dicaproyl phosphatidylserine interacts with rFVIII, causing subtle changes in the tertiary and secondary structure of the protein. Sandwich enzyme-linked immunosorbent assay studies indicate that dicaproyl phosphatidylserine probably interacts with the phospholipid binding region of the C2 domain. The immunogenicity of FVIII-dicaproyl phosphatidylserine complexes prepared at concentrations above and below the critical micellar concentrations of the lipid were evaluated in hemophilia A mice. Our results suggest that micellar dicaproyl phosphatidylserine may be useful to reduce the immunogenicity of rFVIII preparations.

KEYWORDS: Hemophilia A, inhibitor development, aggregation, recombinant human factor VIII, protein folding, factor VIII-DCPS complex

INTRODUCTION

Factor VIII (FVIII) is a multidomain glycoprotein that comprises 6 main domains: NH₂-A1-A2-B-A3-C1-C2-COOH.¹ The protein is synthesized mainly in the liver cells as a 2351

residue single-chain precursor protein.² Prior to secretion, the protein is proteolytically processed to form the heavy chain (A1-A2-B) having a molecular weight of 90 to 180 kDa and the light chain (A3-C1-C2) having a molecular weight of ~80 kDa.³⁻⁵ The heavy and the light chains are held together by a divalent calcium ion.⁶ The protein circulates in the plasma as a complex with von Willebrand factor.⁷⁻⁹

Hemophilia A is a life-threatening bleeding disorder caused by deficiency or dysfunction of FVIII.¹⁰ Hemophilia A patients usually experience spontaneous bleeding that can be controlled only by the exogenous administration of FVIII preparations.¹¹ Currently, replacement therapy with recombinant FVIII (rFVIII) preparations is the treatment of choice for controlling the bleeding episodes. However, replacement therapy with rFVIII preparations leads to the development of inhibitory antibodies in 15% to 30% of patients.¹² The pathophysiology of inhibitor development is not completely understood, with genetic and product-related factors being implicated as contributors to the development of inhibitors.^{13,14} Development of inhibitors complicates replacement therapy with rFVIII preparations, necessitating more frequent and higher doses to control the bleeding episodes. Alternative treatment strategies such as bypass therapy with factor VIIa¹⁵ or porcine FVIII,¹⁶ and immune tolerance therapy with high doses of rFVIII,¹⁷ have been used to manage patients with inhibitors. However, these alternatives are not successful in all patients and significantly increase the cost of therapy. Hence, rFVIII preparations that prevent the development of inhibitors are desirable.

The main immunodominant epitopes of rFVIII have been found to be located in the C2, A3, and A2 domains of FVIII.^{12,18-20} Additionally, the C2 domain region 2291 to 2330 has been shown to encompass several universal immunodominant epitopes for CD4+ T cells.^{21,22} The phospholipid binding region 2303 to 2332 is also located in this area and has structural features of universal immunodominant epitopes.²³

We have previously shown that O-phospho-L-serine,²⁴ the head group of phosphatidylserine and phosphatidylserine-rich liposomes (K. Ramani, V. Purohit, R.M. Straubinger, S.V. Balasubramanian, unpublished data, 2005) capable of binding to the phospholipid binding region, can reduce the immunogenicity of rFVIII in

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hemophilia A mice. Dicaproyl phosphatidylserine (DCPS) is a unique water-soluble lipid that can exist as micelles at concentrations above the critical micellar concentrations (CMCs). Both the soluble and micellar forms of DCPS may interact with rFVIII because of the presence of the phosphoserine head group and influence the immunogenicity of rFVIII. Here, we have investigated the effects of DCPS, a short-chain phosphatidylserine capable of existing in the soluble (monomolecular) as well as micellar (multimolecular assembly) forms, on the immunogenicity of rFVIII in hemophilia A mice. The effects of DCPS on the secondary and tertiary structure of rFVIII were investigated using circular dichroism (CD) and fluorescence spectroscopy, respectively. We confirmed that DCPS can bind to the phospholipid binding region of the C2 domain of rFVIII using sandwich enzyme-linked immunosorbent assay (ELISA). We have also evaluated the impact of DCPS on the immunogenicity of rFVIII hemophilia A mice. The results suggest that DCPS may be useful in reducing the immunogenicity of rFVIII.

MATERIALS AND METHODS

Materials

rFVIII was obtained from Baxter Healthcare (Carlsbad, CA). Monoclonal antibodies ESH4 and ESH8 were obtained from American Diagnostica Inc (Greenwich, CT). Activity assays were performed with normal coagulation control plasma and FVIII-deficient plasma purchased from Trinity Biotech (Co Wicklow, Ireland). Platelin L reagent was purchased from BioMerieux (Durham, NC). The activated partial thromboplastin time (APTT) and Bethesda assay was performed using a COAG-A-MATE coagulation analyzer (Organon Teknika Corporation, Durham, NC). Diethanolamine, tryptophan, and 30% essentially immunoglobulin-free bovine serum albumin were obtained from Sigma (St Louis, MO). 1,6-Diphenyl-1,3,5-hexatriene was obtained from Molecular Probes (Eugene, OR). p-Nitrophenyl phosphate was purchased from Pierce (Rockford, IL). DCPS and dicaproyl phosphatidylcholine (DCPC) as chloroform solutions were obtained from Avanti Lipids (Alabaster, AL). All buffer salts were purchased from Fisher Scientific (Fair Lawn, NJ) and used without further purification.

Determination of CMC of DCPS

Lipid films corresponding to various amounts (5 μ M-1 μ M) of DCPS were prepared from a chloroform stock solution by evaporating the solvent in a rota-evaporator. The films were reconstituted with Tris buffer (5mM or 1mM CaCl₂, 25mM Tris, and 300mM NaCl, pH = 7) by vortexing to obtain 5, 4.5, 4, 3.5, 3, 2.5, 2, and 1mM DCPS

solutions. Two μ L of a 0.005M, 1,6-diphenyl-2,4,6-hexatriene (DPH) solution in tetrahydrofuran was added to the above solutions, incubated at 37°C for 30 minutes, and stored in the dark for 1 hour. The fluorescence of the samples was measured in a PTI-Quantmaster fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ). Excitation wavelength was set at 360 nm, and emission was monitored at 430 nm. An I-shaped cuvette was used for all fluorescence measurements to minimize any interference due to scattering caused by the presence of micelles. The fluorescence intensities were plotted against the concentration of DCPS, and independent linear regressions were performed on the data above and below the putative CMC. The point of intersection of the 2 independent linear regressions was considered the CMC for DCPS.

Preparation of rFVIII-DCPS Complexes

rFVIII-DCPS complexes were prepared by diluting a concentrated stock solution of rFVIII in buffer comprising 0.05mM (below CMC) or 5mM (above CMC) DCPS in 25mM Tris, 1mM CaCl₂, and 300mM NaCl, pH 7.0. The solution was incubated at room temperature for 30 minutes. rFVIII complexes with DCPC were also prepared as described for rFVIII-DCPS complex by diluting with Tris buffer containing 0.05mM (below CMC) DCPC. For the immunogenicity studies, the solutions were prepared using pyrogen-free water and were sterile filtered prior to use.

Effect of DCPS on Tertiary Structure of rFVIII

A stock solution of DCPS at a concentration of 1mM was prepared in Tris buffer (25mM Tris, 300mM NaCl, and 5mM CaCl₂, pH 7) containing 0.017 μ M rFVIII. Required volumes of the above solution as calculated by the alligation method were added to a 0.017 μ M solution of rFVIII in Tris buffer to obtain various concentrations of DCPS. Each addition was followed by an equilibration period of ~5 minutes before the fluorescence of the sample was measured. The fluorescence measurements of rFVIII in the presence of various concentrations of DCPS were performed using a PTI-Quantmaster fluorescence spectrophotometer (Photon Technology International, Lawrenceville, NJ). Excitation wavelength was set at 285 nm, and fluorescence intensity was monitored at 335 nm for single wavelength measurements. Emission spectra were obtained over a wavelength range of 300 to 400 nm. The fluorescence intensity (F) at 335 nm at a given concentration of DCPS was normalized to the fluorescence intensity of rFVIII (F₀) in the absence of DCPS, to obtain the F/F₀ ratio. The F/F₀ ratio relates to rFVIII and DCPS concentrations by the following equation²⁵:

$$\frac{F}{F_o} = 1 - \frac{F_{\max}}{P_T * 2} * [[K_D + P_T + L_T] - \sqrt{[K_D + P_T + L_T]^2 - 4 * P_T * L_T}] \quad (1)$$

where F_{\max} = maximal change in F/F_o , P_T = total protein concentration, K_D = binding constant, and L_T = total ligand concentration.

The data were plotted as F/F_o versus DCPS concentration (μM) and fitted using WinNonlin (Pharsight, Mountain View, CA) with Equation 1 to obtain estimates for K_D . The above equation assumes a stoichiometry (n) of 1 and a single binding site. Attempts were also made to fit the data to an equation assuming 2 independent binding sites.

Effect of DCPS on Secondary Structure of rFVIII

The effect of DCPS on the secondary structure of rFVIII was studied in a manner identical to that described above. rFVIII was used at a concentration of $0.068\mu\text{M}$, and changes in secondary structure were monitored using far UV CD spectroscopy. CD spectra were acquired using a JASCO-715 spectropolarimeter (Jasco, Inc, Easton, MD) calibrated with d-10 camphor sulfonic acid. The spectra were obtained from 208 to 250 nm, and ellipticity was monitored at 215 nm in a 1-cm quartz cuvette. Furthermore, the CD spectrum of rFVIII was corrected by subtracting the baseline spectrum of the appropriate concentration of DCPS in Tris buffer. The ellipticity (E) at 215 nm at a given concentration of DCPS was normalized to the ellipticity of rFVIII (E_o) in the absence of DCPS, to obtain the E/E_o ratio. The E/E_o ratio relates to the rFVIII and DCPS concentrations by Equation 1. The data were plotted and fitted as described above to estimate K_D .

Sandwich ELISA—Interaction of DCPS With rFVIII

Binding of DCPS to the phospholipid binding region of rFVIII was confirmed by evaluating the ability of DCPS to inhibit the binding of rFVIII to ESH4, an antibody that binds to the phospholipid binding region of the C2 domain. The above was confirmed using the sandwich ELISA protocol described previously.²⁴ DCPS was used at concentrations of 0.5, 2, and 5mM. DCPC is a short-chain water-soluble analog of phosphatidylcholine that has a CMC of 15 to 20mM.²⁶ DCPC was also evaluated for its interaction with the phospholipid binding region, as a control.

Effect of DCPS on Thermal Denaturation of rFVIII

All studies with DCPS were conducted at concentrations below the CMC. The unfolding of rFVIII upon thermal stress was monitored using CD spectroscopy. CD spectra were acquired on a JASCO-715 spectropolarimeter cali-

brated with d-10 camphor sulfonic acid. The protein was subjected to thermal denaturation in the presence and in the absence of 0.05mM DCPS at a controlled heating rate of 60°C/hr from 20°C to 80°C and a 2-minute holding time at every 5°C increment. Temperature was controlled using a Peltier 300 RTS unit (Jasco, Inc), and the thermal unfolding profiles were generated using software provided by the CD instrument manufacturer. The data were represented as change in ellipticity ($\delta\theta$) as a function of temperature. $\delta\theta$ was computed as $\theta_t - \theta_n$, where θ_n is the ellipticity of the protein in the native state obtained by averaging the ellipticities at the initial temperatures, and θ_t is the ellipticity at a given temperature. The transition temperature (T_m) for the unfolding profile was obtained by fitting the data to a sigmoid function (Equation 1) using WinNonlin.

$$Y_{\text{observed}} = Y_{\text{native}} - \frac{(Y_{\text{native}} - Y_{\text{unfolded}}) \times T^Y}{T^Y + Tm^Y} \quad (2)$$

where Y_{observed} is the ellipticity at 215 nm at a given temperature, Y_{native} is the ellipticity of the native protein, Y_{unfolded} is the ellipticity of the unfolded protein, T_m is the transition temperature, and γ is the fitting function.

Protein concentrations used were $\sim 20 \mu\text{g/mL}$ in Tris buffer. The spectral changes of the sample were monitored at 215 nm, and the spectra were obtained from 250 to 208 nm in a 1-cm quartz cuvette. The CD spectrum of rFVIII was corrected by subtracting the baseline spectrum of 0.05mM DCPS buffer. Higher concentrations of DCPS contributed significantly to the buffer baseline and were not investigated. The above thermal denaturation studies were conducted in Tris buffer because of its low metal ion binding capacity.²⁷ Because of the high temperature coefficient of Tris buffer, the observed changes at elevated temperatures could be due to a combination of temperature and pH changes. However, thermal denaturation of rFVIII was also carried out in morpholinepropane sulfonic acid (MOPS) buffer, which has minimal pH change at elevated temperatures, and the profiles were comparable to those obtained in Tris.²⁸

Animals

A colony of hemophilia A mice (C57BL/6J with a target deletion in exon 16 of the FVIII gene) was established with breeding pairs from the original colony.²⁹ Equal numbers of adult male and female mice, aged 8 to 12 weeks, were used for the studies. The sex of the animal has no impact on the immune response.³⁰

Immune Response

Immunization of hemophilia A mice consisted of 4 weekly subcutaneous (sc) injections of rFVIII or rFVIII-DCPS complex. Each dose consisted of $2 \mu\text{g}$ of protein in $100 \mu\text{L}$

of Tris buffer. The sc route of immunization was used mainly to amplify the immune response against administered rFVIII.²⁴ Blood samples by cardiac puncture were obtained from immunized animals in the acid citrate dextrose buffer at 6 weeks postimmunization. The immunogenicity of rFVIII-DCPC complex was also evaluated as an additional control to evaluate the specificity of observed effects. All studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University at Buffalo.

Measurement of Total and Inhibitory Anti-rFVIII Antibody Titers

Antibody titers were determined by standard antibody-capture ELISA, as described previously.²⁴ Inhibitory antibody titers were determined using the Nijmegen modification of the Bethesda (NMB) assay, as described previously.²⁴ The inhibitory titers were expressed in terms of Bethesda units (BU); 1 BU is the amount of antibody neutralizing 50% of FVIII activity under prescribed test conditions. Higher inhibitory titers usually indicate higher concentrations of inhibitory antibodies.

RESULTS AND DISCUSSION

CMC of DCPS

The CMC of DCPS was determined by fluorescence spectroscopy using DPH as a hydrophobic probe.³¹ At lipid concentrations below CMC, DPH exists predominantly in an aqueous environment and exhibits low fluorescence. Formation of micelles results in the preferential partitioning of the DPH into the hydrophobic regions of micelles with a concomitant sharp increase in fluorescence. As can be seen in Figure 1, an increase in the concentration of DCPS beyond 2.3mM (estimated using regression, as described in Materials and Methods) results in a sharp increase in fluorescence intensity, indicating the formation of micelles. At concentrations above CMC, formation of a flocculated precipitate was also observed, possibly because of the interaction of DCPS with Ca²⁺. This is consistent with the observation that Ca²⁺ ions interact with phosphatidylserine to form nonlamellar lipidic structures such as chochleates.^{32,33} Reduction in the Ca²⁺ ion concentration to 1mM prevented the formation of the precipitate without altering the CMC (~2.7mM) of DCPS. Hence, subsequent experiments with DCPS at concentrations above the CMC were conducted in buffer containing 1mM CaCl₂.

Effect of DCPS on Tertiary and Secondary Structure of rFVIII

The tertiary structural changes of rFVIII were monitored by steady-state fluorescence spectroscopy in the presence and

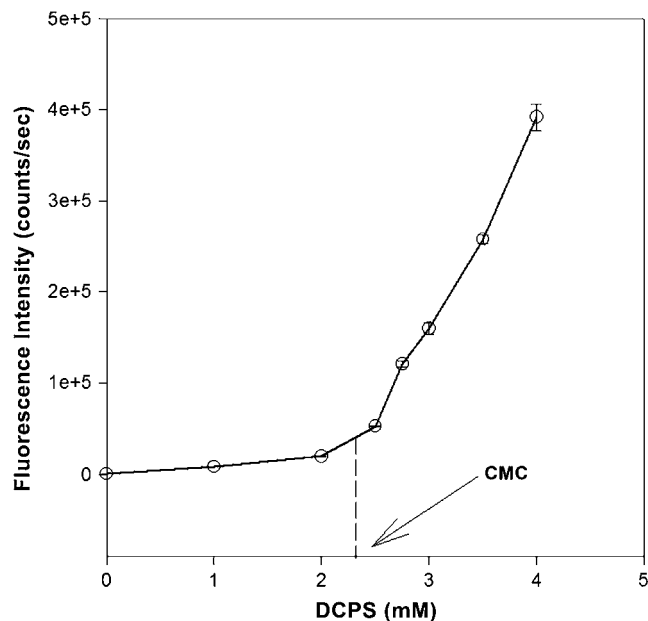


Figure 1. Determination of the critical micellar concentration of DCPS using DPH as a probe. DCPS indicates dicaproyl phosphatidylserine; DPH, 1,6-diphenyl-1,3,5-hexatriene; CMC, critical micellar concentration.

absence of DCPS. As can be seen in Figure 2a, increasing concentrations of DCPS caused a decrease in the intrinsic fluorescence of rFVIII at 335 nm. The decrease in intrinsic fluorescence saturated at a DCPS concentration of ~50μM, but this decrease was not accompanied by a shift in the λ_{max} (Figure 2a). The addition of DCPS did not quench the fluorescence of free tryptophan in solution (Figure 2b). The data suggest that DCPS causes subtle changes in the tertiary structure of rFVIII and are consistent with the observation that DCPS causes similar changes in the tertiary structure upon interaction with factor V_a.³⁴ The data were appropriately described by Equation 1, which estimated an apparent K_D of 3.31μM (±SE, 0.38) with a maximal change (F_{max}) of ~32%. The above data further suggest that the interaction of DCPS with rFVIII is similar to its interaction with O-phospho-L-serine (OPLS), as reported earlier.²⁴ Hence, the interaction of soluble forms of phosphoserine (DCPS below CMC, and OPLS) is mainly mediated by the phosphoserine moiety, with very little contribution from the lipid component of the molecule.

The effect of DCPS on the secondary structure of rFVIII was monitored using far UV CD spectroscopy. Because we were unable to scan the far UV CD spectra beyond 205 nm (because of buffer interference), the exact effect of DCPS on the secondary structural elements (α-helix, β-sheet, etc) of rFVIII could not be ascertained unambiguously. However, structural changes were determined by monitoring the changes in ellipticity at 215 nm in the presence and absence of DCPS. Increasing concentrations of DCPS caused a slight decrease in the negative ellipticity; this suggests

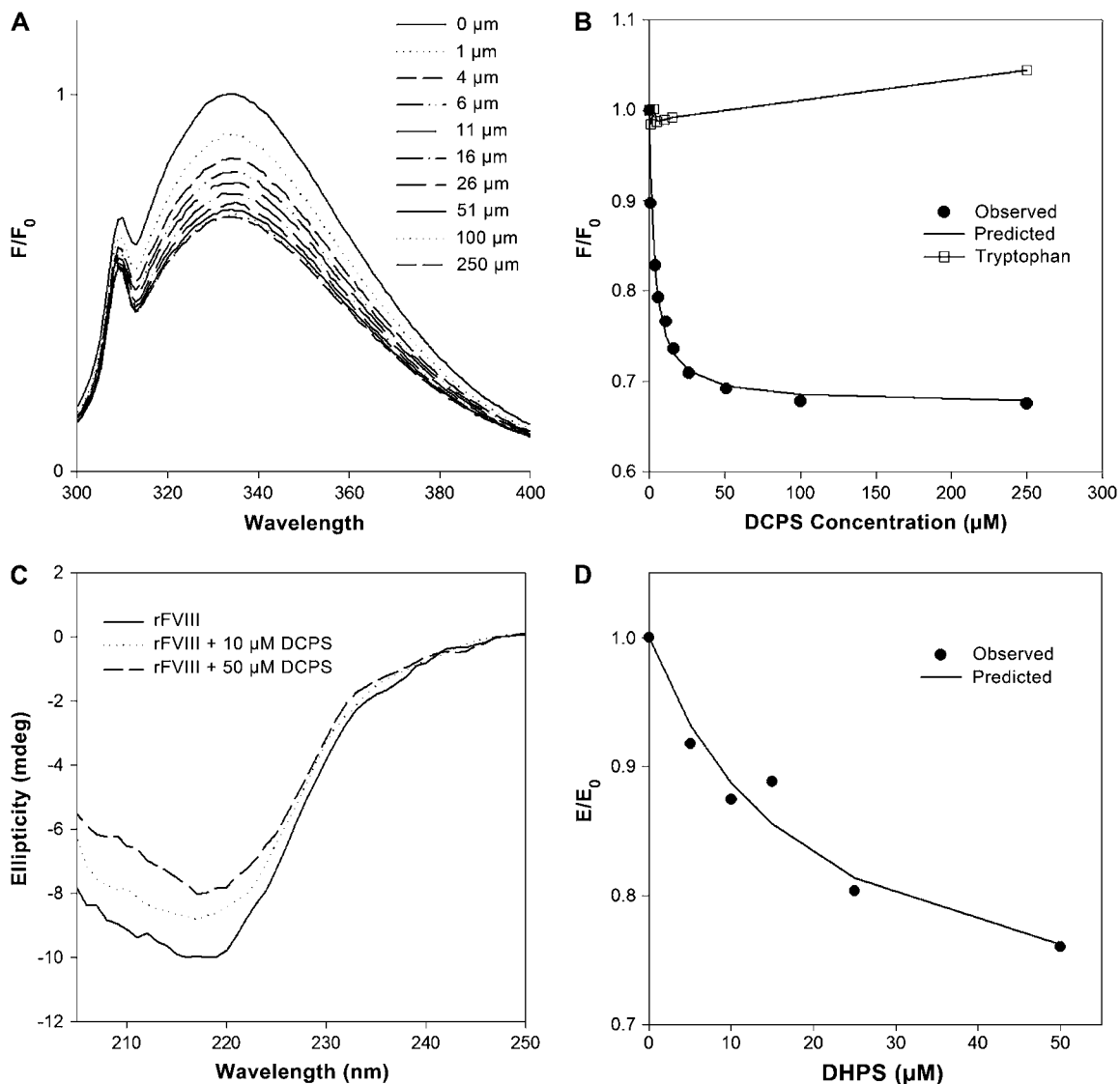


Figure 2. Effect of DCPS on structure of rFVIII. (a) Changes in the emission spectra of rFVIII as a function of DCPS concentration. The sample was excited at 285 nm, and the emission spectra were obtained from 300 to 400 nm. (b) Changes in the intrinsic fluorescence (F/F_0) of rFVIII and tryptophan as a function of DCPS concentration. The excitation was set at 285 nm, and emission was monitored at 335 nm with a 4-nm slit width. The protein concentration typically used was 0.017 μM . Tryptophan was used at a concentration of 0.6 μM . Raw data for rFVIII were fitted using Equation 1 to obtain estimates for the binding parameters. (c) Changes in the far UV-CD spectra of rFVIII obtained from 205 to 250 nm as a function of DCPS concentration. (d) Changes in the ellipticity (E/E_0) at 215 nm for rFVIII as a function of DCPS concentration. The protein concentration typically used was 0.064 μM in a 1-cm pathlength cuvette. Raw data were fitted using Equation 1 to obtain estimates for the binding parameters. DCPS indicates dicaproyl phosphatidylserine; rFVIII, recombinant factor VIII; CD, circular dichroism.

subtle changes in the secondary structure of the protein. The data were fit into Equation 1, and the apparent K_D was estimated to be 19.2 μM ($\pm\text{SE}$, 6.98) with a maximal change of ~33%. The K_D estimated with CD spectroscopy studies appears to be higher than that estimated with fluorescence spectroscopy. This discrepancy is probably caused by a difference in the structural aspects of the protein being monitored by the respective techniques. The estimated K_D is hence a phenomenological K_D and is representative of the changes occurring in only the structural component being monitored.

Sandwich ELISA—Interaction of DCPS With rFVIII

ESH4, a monoclonal antibody, binds to the phospholipid binding region of rFVIII. Binding of DCPS to this region would compete with ESH4 binding, resulting in a lower ELISA response. As can be seen from Figure 3a, increasing concentrations of DCPS caused a decrease in the binding of rFVIII to ESH4, and this decrease was observed for concentrations both above and below the CMC. This indicates that both the soluble and the micellar forms of DCPS can interact with the phospholipid binding region of rFVIII. As

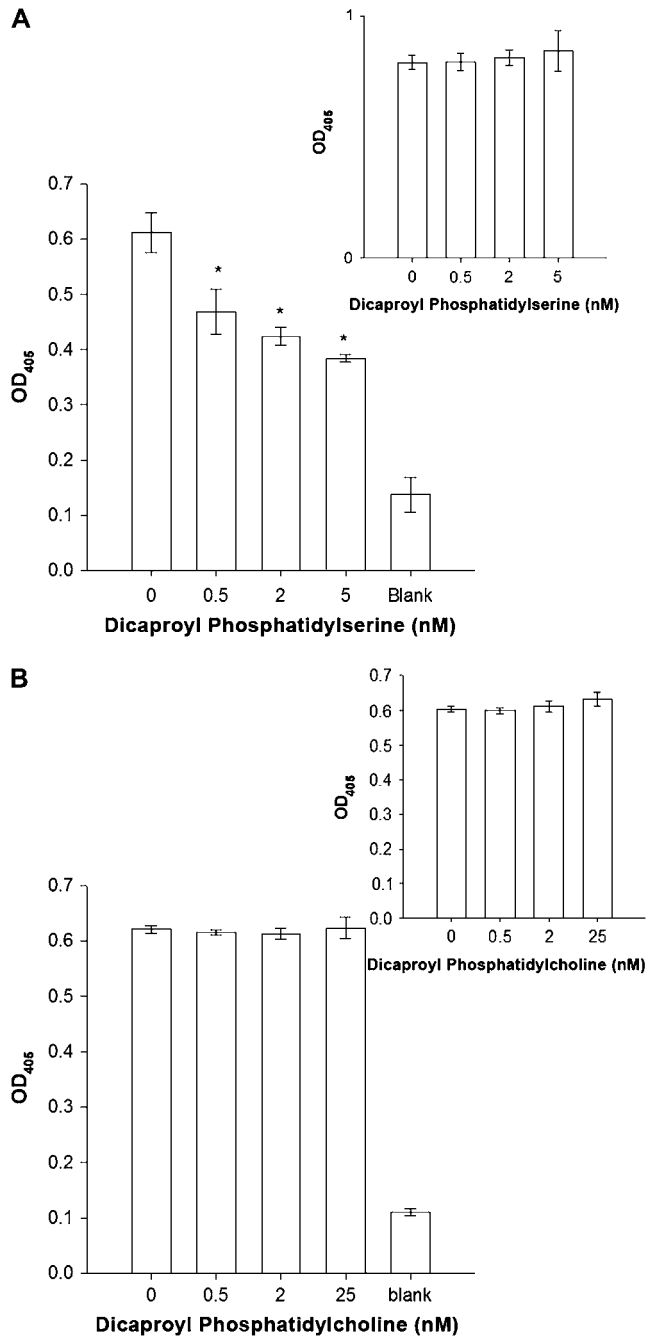


Figure 3. Binding of DCPS to the phospholipid binding region: effect of (a) DCPS and (b) DCPC on the binding of rFVIII to ESH4 as determined by sandwich enzyme-linked immunosorbent assay. Insets: effect of preincubation of DCPS and DCPC on the binding of rFVIII with ESH4. Statistical analysis: analysis of variance followed by post hoc Dunnett test (*, $P < .05$). DCPS indicates dicaproyl phosphatidylserine; DCPC, dicaproyl phosphatidylcholine; rFVIII, recombinant factor VIII.

DCPS has detergent-like properties, an additional control experiment was conducted in which DCPS alone was incubated with ESH4-coated wells before the addition of rFVIII. As can be seen from the inset of Figure 3a, the optical densities were independent of the concentration of DCPS, which rules out the possibility of stripping of the immobilized

ESH4 by DCPS. Hence, the observed reduction in rFVIII binding to ESH4 in the presence of DCPS is mainly due to competition between ESH4 and DCPS for binding to the phospholipid binding region of rFVIII.

The interaction of DCPC to the phospholipid binding region was also investigated in the sandwich ELISA at concentrations above and below the CMC. As can be seen in Figure 3b, DCPC did not interfere with the binding of rFVIII to ESH4. This suggests that DCPC does not interact with the phospholipid binding region of rFVIII and the interaction of DCPS with rFVIII is specific.

Effect of DCPS on Thermal Denaturation of rFVIII

Our previous studies have shown that rFVIII aggregation may at least in part be initiated by conformational changes in the phospholipid binding region in the C2 domain of rFVIII.³⁵ Because DCPS binds to the phospholipid binding region, we determined the effect of DCPS on the aggregation behavior of rFVIII using CD under thermal denaturing conditions. Thermal stress was chosen to investigate the effects of DCPS, as it is frequently used to understand protein folding and stability issues.³⁶

The thermal unfolding of rFVIII at a heating rate of 60°C/hr in the presence and absence of DCPS was monitored at 215 nm using far UV-CD spectroscopy. As can be seen for rFVIII (Figure 4, inset), there was no significant change in

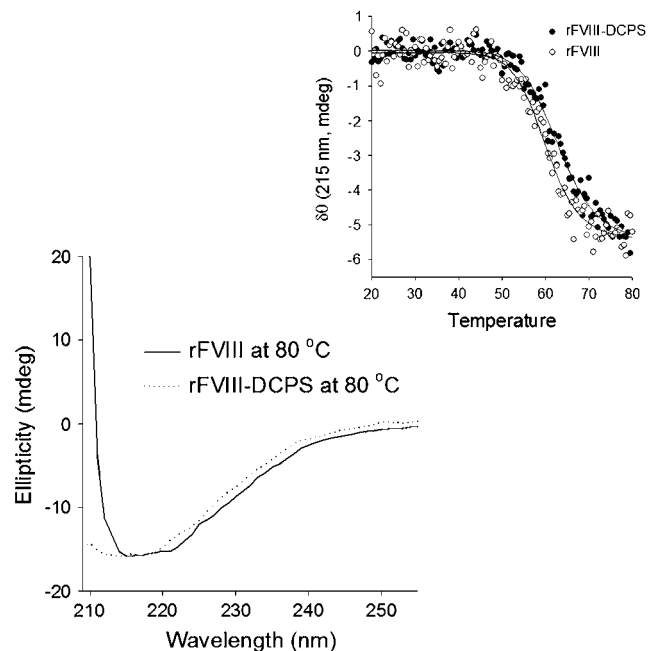


Figure 4. Effect of DCPS on thermal denaturation of rFVIII: far UV-CD spectra of rFVIII and rFVIII-DCPS after heating to 80°C. Inset: Thermal unfolding profiles of rFVIII in the presence (•) and absence (○) of DCPS. The protein concentration was ~20 µg/mL, and DCPS was used at a concentration of 0.05mM. DCPS indicates dicaproyl phosphatidylserine; rFVIII, recombinant factor VIII; CD, circular dichroism.

the ellipticity values over the temperature range of 20°C to 45°C. Beyond 50°C an increase in negative ellipticity was observed. The midpoint of the transition as determined by fitting to Equation 2 was ~60°C. In the presence of DCPS, a similar profile was obtained, suggesting that the intrinsic stability of rFVIII was unaltered.

Far UV-CD spectra were also acquired at various temperatures along the thermal unfolding profile of rFVIII in the presence and absence of DCPS. The far UV-CD spectrum of rFVIII (Figure 2c) acquired at 20°C showed a broad negative band at 215 nm, suggesting that the protein exists predominantly in a β -sheet conformation. At elevated temperatures the spectrum showed (Figure 4) a bathochromic shift in the negative band at 215 nm with a positive band beyond 210 nm, indicating the formation of aggregates stabilized by intermolecular β -strands.³⁵ However, in the presence of DCPS the far UV-CD spectral features characteristic of intermolecular β -strands were not observed (Figure 4), indicating that DCPS interfered with the aggregation of the protein by preventing conformational transitions necessary for the formation of intermolecular β -strands.

Immunogenicity of rFVIII-DCPS Complex

DCPS interacts with the C2 domain of rFVIII, which encompasses several immunodominant epitopes.^{21,22} Hence, we tested the hypothesis that administration of rFVIII complexed to DCPS could reduce the overall immunogenicity of rFVIII in hemophilia A mice. The hemophilia A mice have enabled investigations of the immune responses against rFVIII, as the immune response observed in this animal model has been shown to be qualitatively similar to that observed in humans.^{30,37} Additionally, the murine hemophilia A model has been employed to test strategies that could reduce the immunogenicity of rFVIII.^{38,39} Immunogenicity studies were conducted following subcutaneous administration of the protein to amplify the immune response for meaningful comparison. The total antibody titers and inhibitory titers in immunized mice were determined by antibody capture ELISA and NMB assay (Figure 5).

rFVIII-DCPS complexes were prepared at DCPS concentrations below the CMC, and their immunogenicity was compared with rFVIII's. The total antibody titers for animals given rFVIII with soluble DCPS below CMC (rFVIII-DCPS_{sol}) and rFVIII alone are shown in Figure 5a. Animals given rFVIII-DCPS_{sol} had slightly lower total antibody titers ($9.40E+03 \pm 1.89E+03$, \pm SEM, $n = 12$) relative to animals given rFVIII alone ($1.32E+04 \pm 2.04E+03$, \pm SEM, $n = 15$). A similar pattern (Figure 5b) was also observed for the inhibitory titers: animals administered rFVIII-DCPS_{sol} had lower inhibitory titers ($5.01E+02 \pm 104$ BU/mL, \pm SEM, $n = 9$) relative to animals given rFVIII alone ($6.90E+02 \pm 78.1$ BU/mL, \pm SEM, $n = 13$). Although the

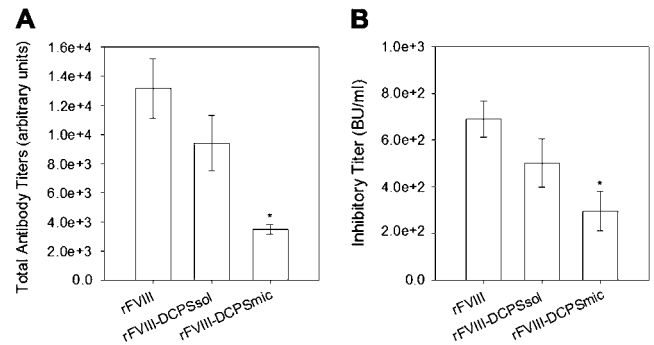


Figure 5. Effect of DCPS on the immunogenicity of rFVIII: (a) total mean antibody titers against rFVIII as determined by antibody capture enzyme-linked immunosorbent assay; and (b) inhibitory antibody titers as determined by Nijmegen modification of the Bethesda assay at 6 weeks in hemophilia A mice immunized with rFVIII, rFVIII-DCPS_{sol}, or rFVIII-DCPS_{mic}. Error bars, \pm SEM. Statistical analysis: analysis of variance followed by post hoc Dunnett test (*, $P < .05$). DCPS indicates dicaproyl phosphatidylserine; rFVIII, recombinant factor VIII; BU, Bethesda unit.

total and inhibitory titers for rFVIII-DCPS_{sol} were lower than those observed for rFVIII, statistical significance could not be achieved. We also evaluated the effect of DCPC (rFVIII-DCPC_{sol}) on the immunogenicity of rFVIII as a control. Animals given rFVIII-DCPC_{sol} had mean total ($9.62E+03 \pm 1.20E+03$ BU/mL, \pm SEM, $n = 12$) and inhibitory ($5.63E+02 \pm 119$ BU/mL, \pm SEM, $n = 8$) antibody titers slightly higher than those observed for rFVIII-DCPS_{sol}, although statistical significance was not achieved. This may suggest that only DCPS is lowering the immunogenicity of rFVIII at concentrations below CMC.

We also investigated the effects of DCPS on the immunogenicity of rFVIII at concentrations (5mM) where DCPS would exist as micelles (rFVIII-DCPS_{mic}). As can be seen in Figure 5, animals given rFVIII-DCPS_{mic} had significantly lower total ($3.51E+03 \pm 332$, \pm SEM, $n = 12$, $P < .05$) and inhibitory (295 ± 84.0 BU/mL, \pm SEM, $n = 8$, $P < .05$) antibody titers relative to animals given rFVIII alone. This suggests that the micellar form of DCPS reduced the immune response against rFVIII.

The reason for the lower immune response observed for rFVIII complexed to micellar forms of the DCPS is not clear. However, we believe that processing of rFVIII by the immune system in the presence of DCPS micelles is inefficient. The inefficient processing can in turn lead to reduced stimulation of the T helper cells, B cells, and concomitant cytokine (IL-10) production that is necessary for sustaining a humoral immune response.

CONCLUSION

Overall, our findings suggest that short-chain phospholipids such as DCPS may be promising excipients for reducing the

immunogenicity of rFVIII and warrant further investigation. The above results further confirm our previous observations, which had suggested that molecules and molecular assemblies capable of interacting with the lipid binding region of rFVIII may be useful multifunctional excipients for rFVIII.^{24,35}

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