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SHORT COMMUNICATION

Determining the Critical Micelle Concentration of a Novel Lipid-Lowering Agent, Disodium Ascorbyl Phytostanyl Phosphate (FM-VP4), Using a Fluorescence Depolarization Procedure

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ABSTRACT

The objective of this study was to determine the critical micelle concentration (CMC) of a novel water-soluble plant sterol derivative (FM-VP4) using a fluorescence depolarization method. The CMC was determined by 1,6-diphenyl-1,3,5-hexatriene (DPH) fluorescence depolarization. Test solutions of various concentrations of sodium dodecylsulphate (SDS) as a positive control or FM-VP4 in water were spiked with 2 μL of 4 mM DPH in tetrahydrofuran (THF) and left overnight to equilibrate in a dark chamber. Fluorescence of each solution was measured at room temperature using a Perseptive Biosystems Cytofluor Series 4000 multi-well plate reader. Fluorescence intensity increases as DPH is incorporated into the hydrophobic core of micelles. Thus, the CMC is the value at which an abrupt increase in intensity is observed. These points were observed at 8 mM and 0.014 mM for SDS and FM-VP4, respectively. Sodium dodecylsulphate was used as a positive control and supports the validity of our results, as the literature values of SDS are reported to be between 8–8.3 mM. The CMC of FM-VP4 is reported to be 0.014 mM.

Key Words: Critical micelle concentration; Novel lipid-lowering agent; Disodium ascorbyl phytostanyl phosphate; Fluorescence depolarization procedure.

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INTRODUCTION

Disodium ascorbyl phytostanyl phosphate (FM-VP4) (Fig. 1) is a semisynthetic esterified phytostanol derivative, produced as the sodium salt form.[1–3] The two major components of FM-VP4 are sodium salts of ascorbyl campestanyl phosphate and ascorbyl sitostanyl phosphate. These molecules comprise a stanyl moiety, linked by an ester bond to a phosphate moiety, which is in turn linked by a second ester bond to an ascorbyl moiety. Recently, our laboratory has reported that FM-VP4 decreases levels of plasma and LDL cholesterol and cholesterol gastrointestinal absorption in vitro and in vivo.[1–3]

Consideration of the molecular structure of the major components indicates coexisting regions of lipophilicity (the stanyl moiety) and hydrophilicity (the ascorbyl phosphate moiety). It is reasonable to propose that such molecules may exhibit certain surface-active (surfactant) characteristics. During development, it was noted that aqueous solutions of the compound tended to foam under certain circumstances (unpublished results), and this behavior is consistent with possession of potential surfactant properties.

It is generally recognized that the concentration-related change from free monomeric surfactant molecules to ordered multimolecular assemblies (micelles) leads to inherent and measurable changes in the colligative properties of their aqueous solutions. Below the critical micelle concentration (CMC), many surfactant molecules exist in the unassociated monomeric state. As their concentration is progressively increased and exceeds the CMC, excess monomers associate into micelles, thus decreasing the overall free energy of the system and increasing its stability.

Certain physical characteristics of surfactant solutions, measured vs. an ascending series of surfactant concentrations, show a noticeable change at the CMC. For example, equivalent conductivity, surface tension, interfacial tension, and osmotic pressure all show a marked inflexion in their observed values when plotted vs. ascending surfactant concentration. The procedure to determine the CMC value of FM-VP4 is based upon the observation that micelles can solubilize hydrophobic molecules, in this case the hydrophobic fluorescence dye probe 1,6-diphenyl-1,3,5-hexatriene (DPH), by entrainment into the micellar structure.[4] A particularly elegant feature of the current method is that DPH demonstrates an increase in fluorescence intensity when present in the hydrophobic core region of the micelle and can therefore be used as a detection analyte to quantitatively indicate any tendency of FM-VP4 to form micelles in aqueous solution.

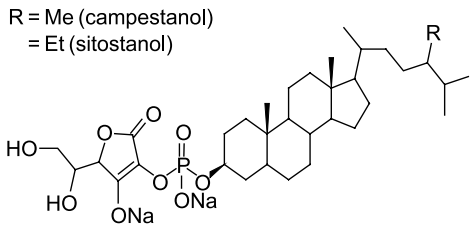


Figure 1. Structure of FM-VP4.

The studies presented in this manuscript were designed to quantitatively investigate whether or not FM-VP4 undergoes micellization with ascending concentration, i.e., to determine if it exhibits a clear CMC value. Information from this study would help us further understand in what physical orientation FM-VP4 is present when incubated in vitro with intestinal cells or when administered to animals.

MATERIALS AND METHODS

Chemicals

FM-VP4 is primarily comprised of two molecular entities, campestanol and sitostanol, each covalently linked to ascorbic acid by a phosphodiester bond (campestanol:sitostanol, 34.62:62.41% w/w) (Fig. 1). The FM-VP4 content will be taken as 97.0% w/w on a dry weight basis (allows for a loss on drying value of 3.00% w/w). For purposes of molarity calculation it will be assumed that minor components are negligible and adjusted material potency comprises the two major components only: 34.84% w/w sodium ascorbyl campestanyl phosphate and 65.16% w/w sodium ascorbyl sitostanyl phosphate. Thus the molarity data presented in Table 1 consists of apparent calculated values, not absolute ones. They, however, will be reasonably accurate for the purpose intended. Sodium dodecyl sulfate (SDS; 99% purity w/w on a dry weight basis, lot no. 78H0702) was purchased from Sigma-Aldrich Canada Limited. A hydrophobic fluorescent dye probe, DPH, (lot no. 84F 1085) was obtained from Sigma-Aldrich Canada Limited. Tetrahydrofuran (THF) was obtained from British Drug Houses Company (BDH) (Toronto).

Determination of Fluorescence Intensity

A DPH stock solution of 10 mM in THF was prepared, and a given volume of DPH solution was added to various concentrations of FM-VP4 or SDS

Table 1. FM-VP4 concentration vs. solution pH.

FM-VP4 test solutions		pH				
mM	mg/mL	Stock 1	Stock 2	Stock 3	Mean	Std dev.
0.144	**0.1**	8.43	8.67	8.71	**8.60**	0.15
0.115	**0.08**	8.27	8.63	8.74	**8.55**	0.25
0.086	**0.06**	8.52	8.32	8.7	**8.51**	0.19
0.072	**0.05**	8.52	8.54	8.59	**8.55**	0.04
0.057	**0.04**	8.41	8.67	8.39	**8.49**	0.16
0.043	**0.03**	8.4	8.59	8.6	**8.53**	0.11
0.036	**0.025**	8.46	8.57	8.52	**8.52**	0.06
0.029	**0.02**	8.38	8.53	8.5	**8.47**	0.08
0.025	**0.0175**	8.44	8.6	8.67	**8.57**	0.12
0.022	**0.015**	8.13	8.44	8.43	**8.33**	0.18
0.018	**0.0125**	8.37	8.46	8.62	**8.48**	0.13
0.014	**0.01**	8.42	8.54	8.54	**8.50**	0.07
0.108	**0.075**	8.31	8.26	8.57	**8.38**	0.17
0.007	**0.005**	8.44	8.31	8.57	**8.44**	0.13
0.004	**0.003**	8.3	8.46	8.59	**8.45**	0.15
0.003	**0.002**	8.31	8.36	8.42	**8.36**	0.06
0.001	**0.001**	7.59	8.61	8.46	**8.22**	0.55

(positive control) solutions. These samples were equilibrated overnight in a dark chamber.[5] Following the overnight equilibration, fluorescence of each solution was measured at room temperature using a Perseptive Biosystems Cytofluor Series 4000 multi-well plate reader. The wavelengths of excitation and emission were 325 nm and 428 nm, respectively. All experimental determinations were carried out at ambient room temperature (23–27°C). The pH of each test solution was measured and recorded, following spectrofluorometric analysis. Each experimental test concentration was run in triplicate, in an ascending concentration series and results were recorded. All solutions were stored at 2–8°C, protected from light, when not in use. Stock solutions were equilibrated to ambient room temperature (23–27°C) prior to serial dilution.

Determination by Transmission Electron Microscopy

Aqueous solutions of FM-VP4 above (0.1 mg/mL) and below (0.01 mg/mL) the critical micelle concentration were visualized for micelle formation using transmission electron microscopy (Fig. 4).

Statistical Analysis

All data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey-Kramer post hoc test using Instat2 (Graph Pad Inc., San Diego, CA). Differences were considered significant if the p value was less than 0.05.

RESULTS AND DISCUSSION

The fluorescence intensity of DPH in solutions of FM-VP4 (Fig. 1) and SDS increased rapidly with increasing concentration (Figs. 2 and 3). Abrupt changes in fluorescence intensities were observed for FM-VP4 (Fig. 2) and SDS (Fig. 3) at concentrations of 0.014 and 8 mM, respectively. As stated previously, since DPH fluorescence intensity increases greatly above the CMC due to its incorporation into the hydrophobic interior of micelles, the abrupt changes of the intensity represent the value of CMC.[4] The CMC of SDS from the fluorescence measurements was found to be 8 mM at ambient temperatures and is in agreement with literature results.[5]

Using this depolarization technique, the CMC of FM-VP4 was found to be 0.014 mM at ambient temperatures. This was confirmed by transmission electron microscopy (Fig. 4A). No FM-VP4 micelles were formed below the CMC (Fig. 4B). Since the major components of FM-VP4 (Fig. 1) indicate coexisting regions of lipophilicity (the stanyl moiety) and hydrophilicity (the ascorbyl phosphate moiety), it is reasonable to suggest that such molecules may

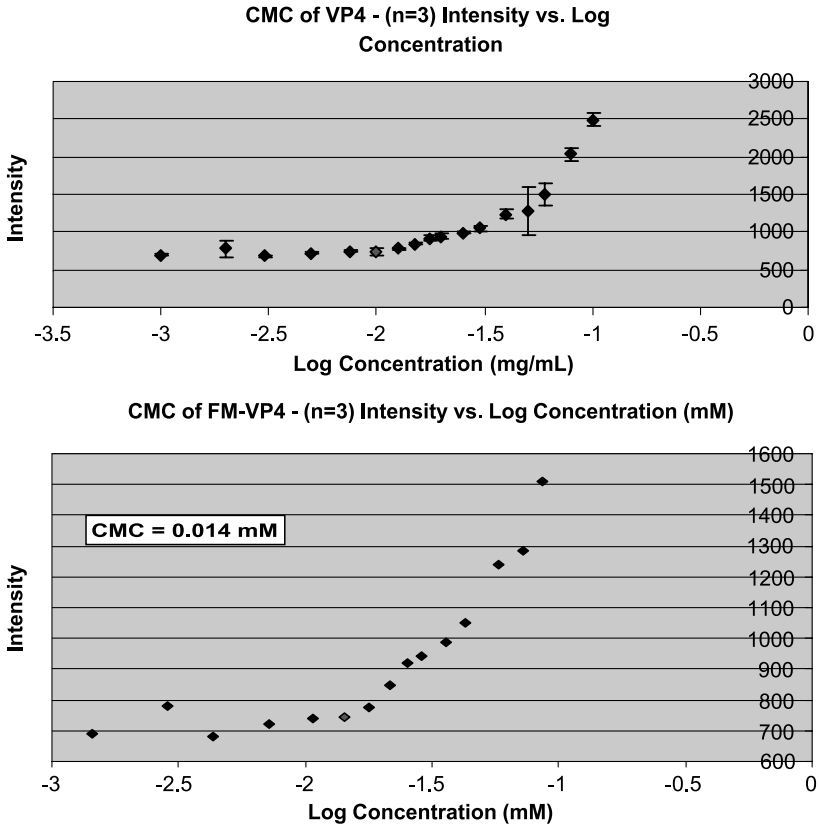


Figure 2. CMC Determination for FM-VP4. Data are presented as mean ± standard deviation (n = 3).

exhibit certain surface-active (surfactant) characteristics. During development, it was noted that aqueous solutions of the compound tended to foam under certain circumstances (unpublished results), and this behavior is consistent with possession of potential surfactant properties. In addition, the pH of various concentrations of FM-VP4 tested remained constant (Table 1). Taken together, these findings suggest that FM-VP4's aqueous solubility may be due to its ability to form micelles, which are soluble within an aqueous solvent (i.e., water) without modifying the solution's pH.

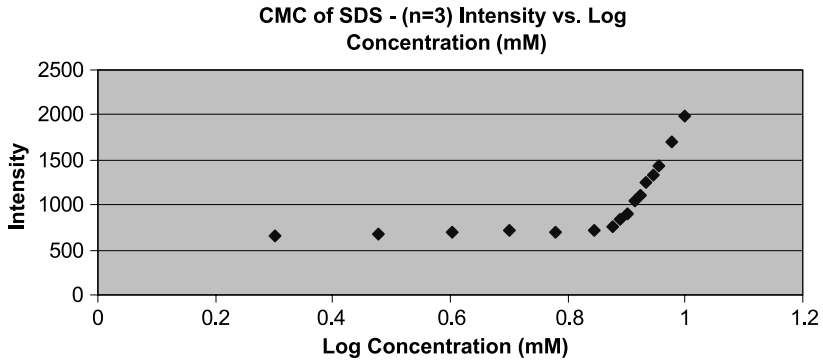
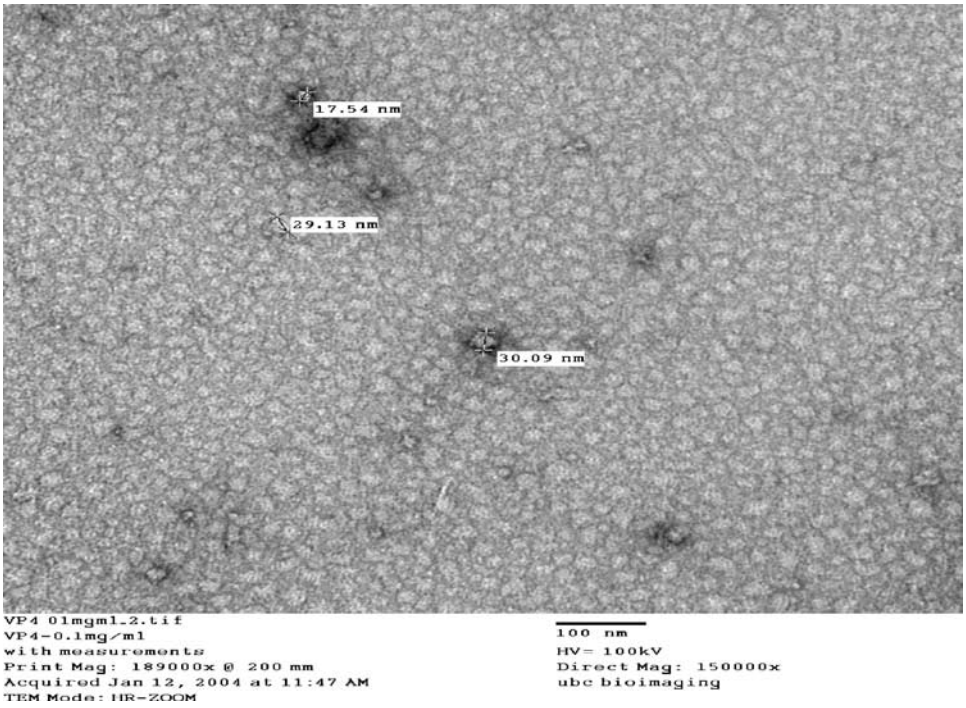


Figure 3. CMC Determination for SDS. Data are presented as mean ± standard deviation (n = 3).

A



B

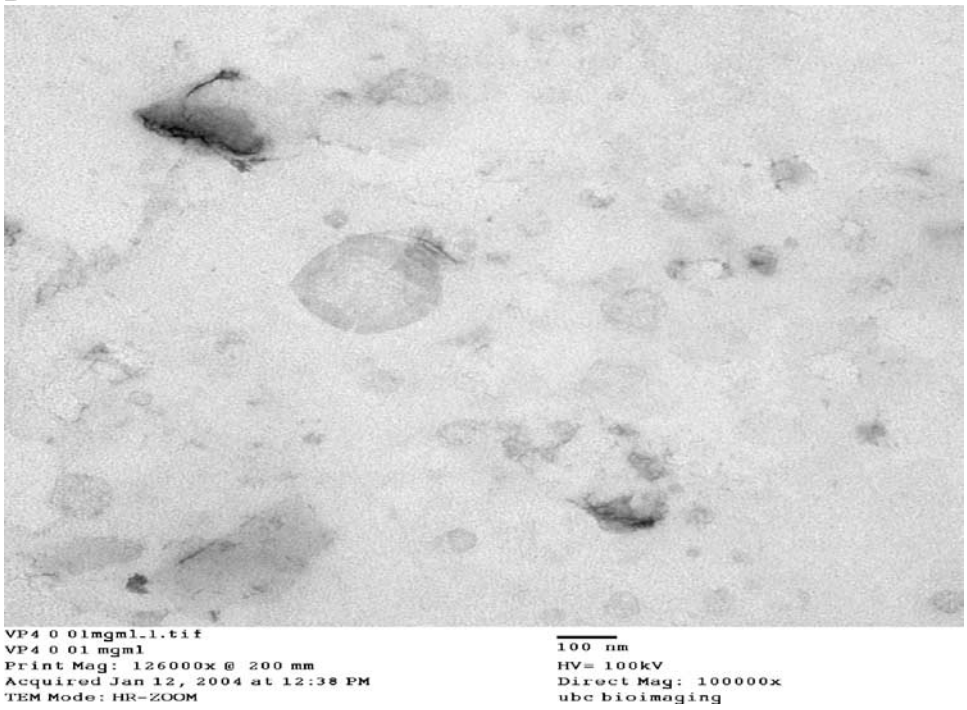


Figure 4. (A) Representative transmission electron microscopy of FM-VP4 dissolved in water above the critical micelle concentration. (B) Representative transmission electron microscopy of FM-VP4 dissolved in water below the critical micelle concentration.

The mode of action of FM-VP4 is still unclear. It acts to rapidly reduce accumulation of cholesterol in cells, likely at the cell surface.[6] However, understanding the physical orientation of FM-VP4 when administered may provide some additional information about its mechanism of action.

CONCLUSIONS

The CMC of FM-VP4 and SDS was determined to be 0.014 mM (Fig. 4) and 8 mM, respectively. No significant change in the pH of the FM-VP4 solution was reported at the concentrations tested. These findings suggest that the aqueous solubility of FM-VP4 is a result of micelle formation.

ACKNOWLEDGMENTS

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