Assessing the Interactions of Auristatin Derivatives with Mixed Phospholipid–Sodium Dodecyl Sulfate Aggregate Dispersions

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ABSTRACT: The aim of this study was to assess what properties of the pseudostationary phases in electrokinetic capillary chromatography affect the interactions between monomethyl auristatin E (MMAE) and hydrophilically modified structural analogues thereof with various lipophilic phases. MMAE is a widely used cytotoxic agent in antibody–drug conjugates (ADC), which are used as selective biopharmaceutical drugs in the treatment of cancers. MMAE and its derivatives are highly lipophilic, yet they fail to interact with biomimicking phosphatidylcholine–phosphatidylserine liposomes. To reveal what properties affect the interaction of the auristatins with cell plasma membrane-mimicking vesicles, capillary electrokinetic chromatography was used with four different types of micellar and vesicular pseudostationary phases: pure vesicles, mixed vesicles, mixed micelles, and pure micelles. Vesicular phases were composed of pure phospholipids [dimyristoylphosphatidylcholine (DMPC) and dilauroylphosphatidylcholine (DLPC)] and phospholipid–surfactant mixtures [sodium dodecyl sulfate, (SDS) with DMPC and DLPC] while the micellar phases comprised pure surfactant (SDS) and surfactant–phospholipid mixtures (SDS–DMPC and SDS–DLPC). In addition, differential scanning calorimetry and dynamic light scattering were used to monitor the aggregate composition. Our data shows that the interaction between hydrophobic auristatin derivatives and hydrophobic pseudostationary phases critically depends on the type, size, and hydrogen bonding capability of the pseudostationary phases.

INTRODUCTION

Antibody–drug conjugates (ADCs) consist of cytotoxic agents linked to monoclonal antibodies and are considered to be promising precision medicines against diseases such as cancer. In more detail, they combine the selective targeting capabilities of monoclonal antibodies with the potent cytotoxicity of the free cytotoxic agent is liberated and thus free to interact once the ADC has reached its destination (i.e., tumor cells), displaying by toxic molecules. It is important to note that the ADC has reached its destination (i.e., tumor cells), the free cytotoxic agent is liberated and thus free to interact with its intracellular targets.1,2 Hydrophilic derivatization of the cytotoxic agents was previously shown to improve the overall properties of ADCs (e.g., the therapeutic window, pharmaceutical efficacy, and issues related to multidrug resistance3,4). Therefore, obtaining information on the biophysical profiles (or hydrophilic character) of the cytotoxic agents is essential to the development of improved ADCs. In recent studies, we investigated the correlation of cytotoxic activity and hydrophobicity in a set of monomethyl auristatin E (MMAE) derivatives and ADCs thereof.5–7 Our studies provided further evidence of the unique properties and advantages of hydrophilically modified MMAE derivatives both in their free form and as final ADCs. During these studies, it was noticed that there was a need for an improved method for assessing the relative hydrophobicity of the auristatins.

Capillary electrophoresis (CE), moreover, capillary electrokinetic chromatography (EKC), is an excellent technique for assessing interactions between compounds and different aggregates. In our previous work,5 we determined the lipophilicity of MMAE and its structural analogues using sodium dodecyl sulfate (SDS) and sodium cholate (SC) mixed micelles. The cytotoxic agents of ADCs are known to affect cell mortality intracellularly, but we demonstrated that they did not interact with standard biomimicking phospholipid vesicles (liposomes), used as a pseudostationary phase in EKC. This was an unexpected observation, considering the lipophilic character of the compounds. The aim of this study was to determine whether this unexpected phenomenon was due to the structural features of the compounds or the type, size, or/and composition of the used lipophilic phase (i.e., the aggregate). In addition to studying the parameters that affect the interactions on their own, the present study was expected to provide useful information on the ability of these systems to accurately model the interactions of the analytes with biological membranes.

Since the appearance of the first micellar EKC (MEKC) application utilizing SDS micelles,8 many pseudostationary phases (PSP) have been used. The PSP can consist of nanoparticles such as nanotubes or graphene,9 ionic...
Figure 1. Schematic illustration of (A) phospholipid membrane saturation and solubilization with surfactants and (B) a simple phase diagram. $c_t^{\text{sat}}$ and $c_t^{\text{sol}}$ correspond to the total surfactant concentration causing membrane saturation and solvation, respectively, at a certain phospholipid concentration. $R_e^{\text{sat}}$ and $R_e^{\text{sol}}$ correspond to the effective surfactant–phospholipid molar ratios causing saturation and solvation of the phospholipid membrane, respectively (slopes in the figure). $c_w^{\text{sat}}$ and $c_w^{\text{sol}}$ are the theoretical surfactant concentrations in water for the onset of saturation and solvation of the membrane, respectively. Illustration adapted from the studies of Lichtenberg et al.\textsuperscript{18} and Majhi et al.\textsuperscript{21}

Figure 2. Molecular structures of the compounds used in the study.
polymers,10 proteins or polysaccharides,11 or liposomes (liposome electrokinetic chromatography, LEKC),12,13 just to mention a few. The PSP for EKC is selected on the basis of the type of analyte under investigation. For example, micelles are suitable for the separation of neutral and hydrophobic drugs, while proteins can be useful for the separation of enantiomers. Moreover, stacking or sweeping methods can be used to improve the sensitivity.11,14,15

Mixed surfactant–phospholipid aggregates have been used to improve the selectivity of hydrophobic analytes, which would co-migrate with common micelles. The most popular mixed aggregates comprise a mixture of neutral (often Brij-35 or Tween 20) and anionic surfactants (often SDS).11,16 Mixed phospholipids–surfactants are also well established. When a surface-active compound is mixed with an aggregate of insoluble amphiphiles, mixed assemblies are formed. The structure depends on the molar ratio of the soluble-to-insoluble amphiphiles. Below a critical saturation ratio (Rsat), the mixed unimers occur as a lamellar phase, and above the critical solvation ratio (Rsol), the lamellar phases are transformed into mixed surfactant–phospholipid micelles. When the phospholipid concentration is kept constant and the surfactant concentration is increased, the surfactant unimers permeate the vesicle (saturation) until a critical saturation concentration (Ce,sat) is reached. Above this value, the mixed phospholipid–surfactant vesicles start to rupture, and mixed vesicles and mixed micelles coexist in the solution. When the surfactant concentration is further increased, a critical solvation concentration (Ce,sol) is reached and only mixed micelles occur in the solution (Figure 1A).17−20 The dependency between the critical surfactant concentrations and the phospholipid concentration can be further illustrated with a phase diagram (Figure 1B).

To assess the hydrophobicity and the interactions of the molecules with biomembrane-mimicking PSPs, MMAE auristatin derivatives (MMAE, derivatives 1, 2, and 3) and four model compounds were studied (shown in Figure 2). The distribution constants were determined using different PSPs. The PSPs were composed of (I) pure phospholipid vesicles ([1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC)], 1,2-dilauroyl-sn-glycero-3-phosphocholine (DLPC), and a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)/1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-di-serine (sodium salt; POPS) mixture, (II) mixed vesicles (SDS with POPC and DLPC with SDS), (III) mixed micelles (DMPC with SDS and DLPC with SDS), and (IV) pure micelles (SDS). In addition, differential scanning calorimetry (DSC) and dynamic light scattering (DLS) were used as complementary techniques to assess and monitor the aggregate type, size, and mixing.

## EXPERIMENTAL SECTION

**Chemicals.** Auristatin derivatives were obtained from Glykos Finland. (Helsinki, Finland). Hydrogen sodium phosphate, thiouria, and the alkyl benzoates (methyl benzoate, ethyl benzoate, propyl benzoate, butyl benzoate, pentyl benzoate, and hexyl benzoate) were purchased from Sigma (Darmstadt, Germany). Naringerin, hesperetin, testosterone, and aldosterone were purchased from Acros Organics (Belgium). Sulfadiazine, naproxen, nadolol, sulfadimethoxine, sulfamethazine, and ketoprofen were kindly donated by Prof. Michael Lämmerhofer (Tübingen University, Germany). POPC, POPS, DLPC, and DMPC were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Methanol (HPLC-grade) and dihydrogen sodium phosphate monohydrate were from Mallinckrodt Baker (Davenport, Netherlands). Sodium hydroxide (0.1 M) was from FF-Chemicals (Yiï-ii, Finland), and sodium dodecyl sulfate (purity 99%) was purchased from Merck (Darmstadt, Germany).

**Sodium Phosphate Buffer Preparation.** Sodium phosphate buffer was used as a background electrolyte (BGE) in CE and as a solvent for all CE, DLS, and DSC samples. The buffer was prepared by mixing appropriate amounts of hydrogen sodium phosphate and dihydrogen sodium phosphate monohydrate to yield an ionic strength (I0) of 10 mM and a pH value of 7.4. The concentrations were determined using the PeakMaster software (available for free at http://web.natur.cuni.cz/flash/) ([Na+] = 7.09 mM and [PO4−3] = 4.14 mM). The buffer was filtered through a 0.45 μm syringe filter (Aireka Scientific, Hang Zhou, China).

**Phospholipid−SDS Dispersion Preparation.** Unilamellar POPC−POPS (80:20 mol %), DMPC, and DLPC liposomes were prepared by measuring a proper volume of the stock solutions (20 mM) in chloroform. The chloroform was evaporated under a stream of air, and the residues were removed by keeping the samples in a desiccator under reduced pressure for 2−4 h. The phospholipids were hydrated to a proper volume of sodium phosphate buffer (pH 7.4, I0 = 10 mM) to yield a sample concentration of 4 mM. The multilamellar liposome dispersion was extruded 19 times through a Millipore (Bedford, MA, USA) 100 nm pore size polycarbonate filter using a Liposo-Fast extruder (Avestin, Ottawa, ON, Canada).

The phospholipid−SDS dispersions in buffer were prepared by mixing appropriate volumes of SDS stock solution in buffer (50 mM) and 4 mM unilamellar liposome dispersion in buffer to yield 1 mM phospholipid concentration and a 2 mM (mixed vesicles) or 6 mM SDS solution (mixed micelles). The dispersions were incubated at room temperature for at least 2 days prior to the measurements to obtain an equilibrium of the unimers.

**Dynamic Light Scattering.** Dynamic light scattering (DLS) measurements were conducted on each surfactant−phospholipid sample prior to the CE runs to confirm the sizes of the aggregates. DLS measurements were performed using a Malvern Zetasizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, U.K.). The concentration of samples containing only liposomes was 0.1 mM. However, using mixed phospholipid−surfactant systems, the phospholipid concentration was kept constant at 1 mM and the SDS concentration was either 2 mM (phospholipid−SDS vesicles) or 6 mM (phospholipid−SDS micelles). A constant temperature of °C was used for all measurements, except for the DMPC measurements. To have DMPC in the fluid phase, all measurements throughout the study were performed at 30 °C, including the DLS measurements. All samples were measured three times (one run consisting of a 10−30 individual measurements) using disposable cuvettes. All sizes are reported as the intensity mean, unless otherwise stated.

**Capillary Electrophoresis.** A Hewlett-Packard 30CE (Agilent, Waldbronn, Germany) instrument was used for all CE runs. A diode array detector with wavelengths of 200, 214, 238, and 270 nm were used for detection. The separation voltage was set at 25 kV, and the capillary cassette temperature was kept constant at 25 °C for POPC−POPS, SDS, and DLS measurements and at 30 °C for DMPC measurements. Uncoated fused silica capillaries (length 30.0/38.5 cm) were from Biotaq (Maryland, USA), and the inner and outer diameters of the capillary were 50 and 375 μm, respectively. MMAE molecules were prepared in methanol (1 mg mL−1), and the samples were diluted with sodium phosphate buffer to gain a final sample concentration of 0.05 or 0.1 mg mL−1. Samples were injected at 10 mbar for 10 s. Thiourea (0.2 mM) was used as an EOF marker, and 10 mM (I0) sodium phosphate buffer at pH 7.4 was used as the BGE solution for CE runs. New capillaries were preconditioned by rinsing for 15 min with 0.1 M sodium hydroxide, 15 min with water, and 10 min (CE) or 2−5 min (MEKC and LEKC) with the BGE solution. All runs were repeated at least five times.

**Differential Scanning Calorimetry.** Microcalorimetry (DSC) measurements were conducted with a VP-DSC Microcalorimeter (MicroCal LLC, MA, USA) to determine the effect of SDS surfactant on the main phase-transition temperature (Tm) of DMPC liposomes. A heating rate of 60 °C h−1 was used within a temperature range from 5 to 40 °C. Unilamellar DMPC liposome dispersions (1 mM) with
Table 1. Physicochemical Properties of the Phospholipids, Surfactants, and Mixed Aggregates

| mixture abbreviation | mixture | occurrence in aqueous phase | \( \epsilon_{\text{membrane-bound surfactants}} \) | \( \epsilon_{\text{free surfactants}} \) | \( \nu_{\text{POPC}} \) (10^-4 mol \( \text{m}^{-3} \text{V}^{-1} \text{s}^{-1} \)) | \( \nu_{\text{spec,vol}} \) (ml g⁻¹) | \( \nu_{\text{mol,vol}} \) (l mol⁻¹) | size (nm) |
|----------------------|---------|-----------------------------|-----------------|-----------------|------------------|-----------------|-----------------|---------|
| 80:20 mol % 0.5 mM POPC−POPS | vesicles | −4.29 | 0.719 | 143 ± 17 |
| 80:20 mol % 1 mM POPC−POPS | vesicles | −4.39 | 0.719 | 143 ± 17 |
| 20 mM SDS | micelles | −4.58 | 0.853 (25 °C) | 0.246 | 2.5 ± 0.2* |
| 1 mM DMPC | vesicles | −0 | 0.978 (30 °C) | 0.663 | 154 ± 10 |
| 1 + 2 | 1 mM DMPC + 2 mM SDS | 1.09 | 0.91 | −4.94 | 0.446 | 135 ± 8.3 |
| 1 + 6 | 1 mM DMPC + 6 mM SDS | 2.54 | 3.46 | −4.03 | 0.364 | 7.6 ± 1.2* |
| 1 mM DLPC | vesicles | −0 | 0.962 (20 °C) | 0.598 | 139 ± 8.4 |
| 1 + 2 | 1 mM DLPC + 2 mM SDS | −4.69 | 0.415 | 137 ± 1.0 |
| 1 + 6 | 1 mM DLPC + 6 mM SDS | −3.74 | 0.345 | 5.4 ± 1.3* |

*The average size is given as the number mean (100% micelles).

and without SDS were degassed under vacuum for ca. 5 min prior to the DSC measurements. The concentrations of SDS varied between 0.5 and 12 mM, and all samples were diluted with sodium phosphate buffer. Three heating and three cooling scans were recorded, and the samples were kept at 5 °C for 30 min prior to the heating scans. All scans were normalized to the total unimer concentration in vesicles \( \epsilon_{\text{lipid + surfactant}} \) utilizing eq 2.

**Calculations of Unimer and Aggregate Concentrations.** To estimate the distribution constants of the compounds into phospholipid−SDS vesicles and micelles, free and bound unimer concentrations of surfactants were determined. When 1 mM liposomes are mixed with 2 mM SDS, mixed vesicles are formed without the formation of mixed micelles (confirmed in this work by DLS), thus the vesicle saturation concentration \( \epsilon_{\text{sat}} \) is not yet exceeded. When the SDS concentration is further increased to 6 mM, a completion of vesicle solubilization \( \epsilon_{\text{sat}} \) is reached, and all phospholipid−SDS aggregates occur as mixed micelles (confirmed by DLS, 100% peak intensity by number mean). The concentration of a membrane-bound surfactant \( \epsilon_{\text{bound}} \) can be determined by eq 1, which is adapted from ref 18.

\[
\epsilon_{\text{bound}} = \epsilon_{w} K \Delta (\epsilon_{w} + \epsilon_{a})
\]  
(1)

The phospholipid concentration \( \epsilon_{w} \), surfactant concentration in water \( \epsilon_{a} \), and partition coefficient \( K \) are known. \( K \) can be further determined by eq 2, where the \( R_{s} \) is the critical surfactant to phospholipid ratio.

\[
K = \frac{R_{s}}{\epsilon_{w} + \epsilon_{a}}
\]  
(2)

The membrane-bound surfactant concentrations for the mixed vesicles \( \epsilon_{w} = 1 \text{ mM for DMPC and } \epsilon_{w} = 2 \text{ mM for SDS at } 30 \text{ °C} \) and for mixed micelles \( \epsilon_{w} = 1 \text{ mM for DMPC and } \epsilon_{w} = 6 \text{ mM for DMPG at } 30 \text{ °C} \) were 1.09 and 2.54, respectively, using parameters from a study of Majhi et al.21 \( (R_{s} = 0.57, \epsilon_{w} = 4.4 \text{ mM, and } \epsilon_{w} = 5 \text{ mM}) \). When the membrane-bound surfactant concentration \( \epsilon_{\text{bound}} \) is subtracted from the total surfactant unimer concentration, the concentration of free unimers can be determined. The results are shown in Table 1.

**Calculations of Retention Factors and Distribution Constants.** The retention factor \( k \) elucidates the ratio of time an analyte is retained in the pseudostationary stationary phase to the time it is retained in the aqueous phase. Herein, \( k \) can be defined as the molar ratio of an analyte incorporated into a PSP and into an aqueous phase \( (\phi_{\text{PSP}}) \), which will depend on the PSP concentration. In EKC, \( k \) values can be calculated using eq 3 when the effective electrophoretic mobilities of an analyte are known with \( \mu_{\text{PSP}} \) and without \( \mu_{a} \) the PSP, and the effective electrophoretic mobility of the vesicles or micelles \( \mu_{\text{lip}} \) is determined.

\[
k = \frac{\mu_{\text{PSP}} - \mu_{\text{aq}}}{\mu_{\text{PSP}} - \mu_{\text{lip}}}
\]  
(3)

An iteration procedure, employing a homologous series of alkylbenzoates, was used for the \( \mu_{\text{PSP}} \) determination as previously reported,22,23 and the resulting values are given in Table 1. The distribution constant \( (K_{D}) \) is the molal concentration ratio of an analyte between a PSP and an aqueous phase, and it is calculated for systems with known phase ratios \( (\phi) \) using eq 4.

\[
K_{D} = \frac{k}{\Phi}
\]  
(4)

The phase ratio elucidates the volume ratio of the PSP and the aqueous phase in the fused silica capillary, and it is calculated with eq 5.

\[
\phi = \frac{V_{\text{PSP}}}{V_{\text{aq}}} = \frac{\nu_{\text{spec,vol}} M_{\text{PSP}} - \text{cmc}}{1 - (\nu_{\text{spec,vol}} M_{\text{PSP}} - \text{cmc})}
\]  
(5)

where \( V_{\text{PSP}} \) and \( V_{\text{aq}} \) are the volumes of the PSP and the aqueous phase in the capillary, respectively, \( \nu_{\text{spec,vol}} \) is the partial specific volume, \( M_{\text{PSP}} \) is the molar mass, \( c_{\text{PSP}} \) is the total concentration, and \( c_{\text{lip}} \) is the critical micelle concentration of the PSP. The term \( c_{\text{PSP}} - c_{\text{cmc}} \) illustrates the total aggregate concentration in the capillary, thus the concentration of the free surfactant unimers in the capillary is subtracted from the total unimer concentration. In the case of mixed liposomes (80:20 mol % POPC−POPS), the phospholipids were assumed to mix totally, and because of the low cmc’s of the phospholipids (in the nanomolar range),24 the cmc of the aforementioned mixture is neglected \( (\text{cmc} \ll \epsilon_{\text{lipid}}) \).

The partial molar volume \( (\epsilon_{\text{mol,vol}}) \), which is the volume that 1 mole of a mixture component occupies in a solution, was calculated from the \( \epsilon_{\text{spec,vol}} \) of the unimer (shown in Table 1) by multiplying the value by the molar mass of the unimer. The molar volumes for the mixtures were obtained by normalizing the \( \epsilon_{\text{mol,vol}} \) of the unimers to the molar ratio of the species in the aggregates \( \epsilon_{\text{mol,vol}} / \epsilon_{\text{tot,aggregate}} \). The \( \epsilon_{\text{spec,vol}} \) of POPC is 0.996 ml g⁻¹,25 whereas the \( \epsilon_{\text{spec,vol}} \) for POPPS was not available in the literature, to the best of our knowledge. The \( \epsilon_{\text{mol,vol}} \) of 0.709 l mol⁻¹ was used for POPPS, and it was approximated to be 0.030 l mol⁻¹ less than the \( \epsilon_{\text{mol,vol}} \) of 1,2-diolooyl-12-dioleoyl-sn-glycerol-3-phosphocholine (DOPC; 0.740 l mol⁻¹).26 The subtracted value was an average of the differences in \( \epsilon_{\text{mol,vol}} \) of DOPC and POPPS as well as DOPG and POPG, illustrating a change of two carbons and a double bond in the palmityl acyl chain. The
physicochemical properties of the analytes. In our previous interactions of MMAE and its derivatives was due to the POPS liposomes were a suitable PSP, and the lack of the distribution constant) increases. This con
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the distribution constants of the positive and negative model analytes were determined. First negatively charged phospholipids were prepared using zwitterionic POPC and negatively charged POPS (80:20 mol %). POPC and POPS were chosen for this study because phosphatidylcholine lipids are the most abundant phospholipids in eukaryotic cells and the total net charge of the membrane lipids is negative. The effective electrophoretic mobilities of the liposomes at concentrations of 0.5 and 1 mM are shown in Table 1. Even though most of the aforementioned compounds and the alkylbenzoates interacted with the liposomes (Figure 3), MMAE and structural analogues 1, 2, and 3 did not have any interactions with the liposomes.
The hydrophobicity of the alkylbenzoates increase as the alkyl chain length is elongated, thus the interaction (i.e., distribution constant) increases. This confirms that POPC−POPS liposomes were a suitable PSP, and the lack of the interactions of MMAE and its derivatives was due to the physicochemical properties of the analytes. In our previous study, we assessed the hydrophobicity of MMAE, 1, and 2 using SDS and sodium cholate micelles. All compounds were shown to be highly hydrophobic (log $K_D$ SDS−SC:20−40 mM $= 1.6−2.4$), and therefore the compounds were expected to interact with the POPC−POPS liposomes. In order to confirm that the absence of interactions was not related to the small number of PSP aggregates in the capillary, the phospholipid concentration was further increased to 4 mM (data not shown). However, interactions with the liposomes were not found.

**RESULTS AND DISCUSSION**

**Interaction of Compounds with POPC−POPS Liposomes.** To assess the interaction of the analytes with various aggregates of different sizes, types, and unimer compositions, the distribution constants of the positive and negative model analytes were determined. First negatively charged phospholipids were prepared using zwitterionic POPC and negatively charged POPS (80:20 mol %). POPC and POPS were chosen for this study because phosphatidylcholine lipids are the most abundant phospholipids in eukaryotic cells and the total net charge of the membrane lipids is negative. The effective electrophoretic mobilities of the liposomes at concentrations of 0.5 and 1 mM are shown in Table 1. Even though most of the aforementioned compounds and the alkylbenzoates interacted with the liposomes (Figure 3), MMAE and structural analogues 1, 2, and 3 did not have any interactions with the liposomes.

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**Mixed Aggregates.** To scrutinize whether the lack of interaction between the compounds and POPC−POPS (80:20 mol %) liposomes was due to the selection of the unimers (i.e., the phospholipids) or the aggregate size and type (vesicles vs micelles), different mixtures of phospholipids (DMPC and DLPC) and SDS were prepared. DLPC has 12 carbons in the acyl chains, thus it is expected to solubilize SDS having 12 carbons in the alkyl chain as well, better than the other phospholipids. Because the transition temperature of DLPC is $-1.8 ^\circ C$, occurring outside of the operation range of the DSC instrument, DMPC phospholipids, having 14 carbons in the acyl chains and a transition temperature of 23.9 $^\circ C$, were also used in the study. In addition, Majhi et al. have studied the interactions between the DMPC phospholipids and SDS surfactants, thus a phase diagram of the mixture could be utilized.

The solubility of the SDS surfactants in the vesicle bilayer was routinely inspected using the Zetasizer instrument. The sizes of the pure DMPC and DLPC liposomes, shown in Table 1, were $154 \pm 10.4$ and $139 \pm 8.4$ nm, respectively. When 2 mM SDS was added to the liposome dispersions, the sizes decreased slightly to $135 \pm 8.3$ and $137 \pm 2.0$ nm, respectively; however, no micelles occurred. The results correlate with a previous study in which the addition of charged surfactants to phosphatidylcholine liposomes has been shown to decrease the hydrodynamic radius resulting from the reduction in the electrostatic charge of the headgroups of amphiphilic phospholipids that cause bilayer condensation. When the SDS concentration was further increased to 6 mM, the mixed vesicles were disrupted and most of the aggregates (100% peak intensity by number mean − 84% peak intensity by intensity mean) occurred as mixed phospholipid−surfactant micelles in the dispersion. Large particles scatter more light than small particles; therefore, the light-scattering measurements are biased toward larger particles. When the average size is reported as the intensity mean, the light scattered from large particles mainly determines the scattering intensity, whereas
when the size is reported as the number mean the light-scattering intensity is normalized to the number of particles. Therefore, when the size distribution is reported as the number mean of the particles, it correlates to the number of existing particles (100% micelles in this case) and gives a better picture of the small aggregates in the solution.

**Differential Scanning Calorimetry.** To survey the formation of surfactant—phospholipid aggregates, differential scanning calorimetry was used. DSC measures the thermodynamic properties of thermally induced transitions in substances, which require or release energy. These include conformational changes in macromolecules, binding to proteins, and so forth. In liposomes, the main phase transition (i.e., the chain-melting transition) occurs when the highly ordered crystalline solid state \((L_β)\) is transformed into a disordered liquid state \((L_α)\). The main transition of the DMPC liposomes decreased as expected when 0.5 and 1 mM SDS were added to the dispersion, as shown in Figure 4.

When the concentration was further increased to 2 mM, the \(T_m\) increased again to its original position, indicating a...
stabilization of the liposome bilayer. This sort of rise in the $T_m$ has been shown before with DPPC–fatty acid complexes (palmitic, myristic, and steric acids) and DMPC–myristic acid complexes when the gel phase is transformed into fluid or into some other phase (e.g., inverse hexagonal $H_I$ phase or isotropic I phase) seen from their phase diagrams. A common feature for the DPPC–fatty acids is that for a stoichiometry of 1:2 a so-called molecular compound (phase compound) exists, with an unexpectedly high heat of melting, when the gel phase is transformed into fluid or into some other phase (e.g., reverse hexagonal $H_I$ phase or isotropic I phase) seen from their phase diagrams. A common feature for the DPPC–fatty acids is that for a stoichiometry of 1:2 a so-called molecular compound (phase compound) exists, with an unexpectedly high heat of melting, when the gel phase is transformed into fluid or into some other phase (e.g., reverse hexagonal $H_I$ phase or isotropic I phase) seen from their phase diagrams. A common feature for the DPPC–fatty acids is that for a stoichiometry of 1:2 a so-called molecular compound (phase compound) exists, with an unexpectedly high heat of melting, when the gel phase is transformed into fluid or into some other phase (e.g., reverse hexagonal $H_I$ phase or isotropic I phase) seen from their phase diagrams.

As expected a further increase of SDS to 6 mM, which is above the vesicle saturation point, decreased the peak size because a vesicle to micelle transition decreases the number of bilayers undergoing gel–fluid transitions. However, a small peak was observable, indicating that some vesicles were still occurring in the dispersion, as seen from the DLS data as well. Finally, at an SDS concentration of 12 mM no vesicles occurred in the dispersion as evidenced by the lack of endotherm. At this concentration, all of the phospholipid–SDS aggregates occurred as micelles.

Interaction of Analytes with Phospholipid–SDS Aggregates. The distribution constants of the model compounds and the auristatin derivatives (MMAE, 1, 2, and 3) are presented in Figure 5.

The distribution constants of all model compounds (negatively charged naringerin and hesperetin as well as uncharged testosterone and aldosterone) obtained using the four different PSPs were in a good agreement with each other. The distribution between the neutral analytes (testosterone and aldosterone) and the zwitterionic liposomes (DMPC and DLPC) could not be detected with this method because of the lack of moving components in the capillary. In addition, naringerin and hesperetin could not be detected in the presence of pure DMPC because of an interfering system peak.

Negatively charged test analytes naringerin and hesperetin, with molar masses 2.4–3.3 times smaller than the molar masses of the auristatin derivatives, had the largest distribution constants when phospholipid–SDS vesicles were used as a PSP. This confirms that phospholipid vesicles serve as a good biological model for cell membranes and can be used for interaction studies for relatively small compounds (MW < 700 $g mol^{-1}$). Neutral testosterone and aldosterone, on the other hand, had the highest distributions in the micellar aggregates, SDS, and SDS–phospholipid micelles, confirming that the analyte type (size, charge, electronegativity) affects the distribution between an aqueous and hydrophobic phase.

The original aim was to study the effect that the increased hydrophilicity of auristatin derivatives has on their interactions with biomimicking membranes. In this study, auristatin derivatives containing carbohydrates were utilized, thus MMAE was expected to be the most hydrophobic compound. All of the auristatin derivatives had stronger interactions with the micelles than with the vesicles. MMAE had the largest $K_D$ value of the used analytes, as expected, and the distribution into the different PSPs decreased as follows: SDS–phospholipid micelles > SDS micelles > DMPC vesicles > mixed vesicles. The order was similar to that for structural analogues 2 and 3, while structure 1 had the strongest distribution into SDS micelles. Compound 1 has the lowest theoretical distribution coefficient ($log D$ values at pH 7.4 were 2.0, −0.43, 1.1, and 1.3 for MMAE, 1, 2, and 3, respectively), being the least lipophilic compound. In our previous study, SDS was shown to have poor selectivity toward the auristatin derivatives as a result of their nearly complete solubilization into the SDS micelles. SDS micelles are stronger hydrogen bond donors than 1-octanol; therefore, they are expected to have strong interactions with hydrogen bond acceptor solutes such as MMAE and its derivatives. However, because MMAE, 2, and 3 had the strongest interactions with micelles comprising phospholipids and SDS, it seems that phospholipids solubilize these compounds even better than pure SDS. Yet, because the interactions between the compounds and the vesicles containing phospholipids and SDS had negligible interactions with the compounds, it is inevitable that the aggregate type has a major effect on the distribution of the compounds between the hydrophobic and aqueous phases. Moreover, because the interactions decreased as SDS–phospholipid micelles > SDS micelles > DMPC vesicles > mixed vesicles for most of the auristatin derivatives, it should be noted that the interactions were not solely concentration-dependent. The aggregate–analyte interaction was more dependent on the change in the aggregate type than on the concentration of SDS (increasing the SDS concentration did not gradually increase the interactions). Thus, it is of importance to know the aggregate type (micelles vs vesicles) when mixed aggregates are formed in a solution and interactions are studied.

The difference in the $K_D$ values for MMAE and its structural analogues was tens of thousands ($log K_D$ varied between 0 and 4.5), while the difference of the $K_D$ values for the model compounds was only a few thousands ($log K_D$ values varied between 1.4 and 3.5). This indicates that even a small modification in the molecular structure of MMAE has a significant effect on its distribution properties. This confirms that a small hydrophilic modification of auristatins decreases their lipophilicity and may be beneficial in circumventing the off-site cytotoxicity displayed by these toxic compounds.

Pure liposomes are not the most suitable pseudostationary phases when the interactions of relatively large and highly lipophilic ($log K_D > 3$) compounds are assessed. Here we show that surfactant micelles and mixed micelles are better pseudostationary phases for the interaction studies with these types of compounds. Furthermore, these results indicate that the selection of the PSP composition and the aggregate type and size are highly relevant in EKC and in other methods, in which mixed aggregates are utilized. Moreover, to get more information on the analyte lipophilicity it is advisable to study more than one type of PSP.

CONCLUSIONS

The interactions of auristatin derivatives with various pseudostationary phases in electrophoretic capillary chromatography...
raphy were assessed to scrutinize which properties of the compounds and the pseudostationary phases affect the interactions. Highly hydrophobic MDAE as well as substrates 2 and 3 had high distribution constants (5000–40 000) when mixed phospholipid–surfactant micelles were used as PSPs; however, they did not interact with negatively charged POPC–POPS (80:20 mol %) liposomes. This is rather surprising as the POPC–POPS liposomes represent the negatively charged eukaryotic cell membrane well. In addition, a set of model compounds was distributed into the POPC–POPS liposomes, thus underlining that the lack of interactions between the auristatin derivatives with the vesicular PSPs was due to the physicochemical properties of these molecules. The tubulin polymerase-inhibiting auristatins are potent cytotoxic agents and are liberated from the ADCs inside cancer cells. Therefore, knowledge of their interactions with cell membranes and a possible escape from the intended cells is important to obtain. Surprised by the initial discovery that the auristatins did not interact with the biomimicking membrane model POPC–POPS, different PSPs needed to be assessed. We chose to focus on four different PSPs in this study. These were pure liposomes (DMPC and DLPC), mixed vesicles (1:2 mM DMPC–SDS and DLPC–SDS), mixed micelles (1:6 mM DMPC–SDS and DLPC–SDS), and pure micelles (20 mM SDS). The PSP interactions of a set of model compounds were relatively similar (𝐾, values of between 500 and 6000); however, the negatively charged compounds preferred to interact with mixed vesicles while the neutral compounds preferred to interact with the SDS micelles. This confirms that the used PSP mixtures were suitable for the interaction studies of analytes with various physicochemical properties. SDS micelles are strong hydrogen bond donors, and they solubilize hydrogen-bond-accepting compounds such as the auristatins utilized in this study well. The highest distribution of the auristatin derivatives was witnessed when mixed micelles were utilized, thus indicating that the existence of phospholipids in the micelles increases the interactions. This, together with the fact that these compounds do not have any interactions with vesicles consisting solely of phospholipids, indicates that the aggregate type has a major effect on the interactions of these highly hydrophobic molecules with the lipid membrane models. Altogether, our results show that the commonly employed POPC–POPS biomimicking membrane model may not have optimal properties when studying the interactions of all types of molecules. In these cases, mixed aggregates may be required in order to study the properties and interactions of the analytes with PSPs. If the interactions are studied using mixed aggregates, then it is very important to know the aggregate type because this was shown to be more important than increasing the concentration of the aggregate components.

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**Notes**

The authors declare no competing financial interest.

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**UNCOMMON ABBREVIATIONS**

*′ sat′, total surfactant concentration causing membrane saturation; ′ sol′, total surfactant concentration causing membrane solubilization; ′ sat′, critical surfactant to phospholipid ratio causing saturation; ′ sol′, critical surfactant to phospholipid ratio causing solubilization; ′ sat′, surfactant concentration causing membrane saturation in water; ′ sol′, surfactant concentration causing membrane solubilization in water

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