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Characterization of Lipid-Based Hexosomes as Versatile Vaccine Carriers

Leticia Rodrigues†, Konstantinos Kyriakos‡, Fabian Schneider‡, Hendrik Dietz‡, Gerhard Winter‡, Christine M. Papadakis‡, and Madlen Hubert†,§,*

†Department of Pharmacy, Pharmaceutical Technology and Biopharmacy, Ludwig-Maximilians-Universität München, Butenandtstraße 5-13, DE-81377 Munich, Germany
‡Physics Department, Soft Matter Physics Group, Technische Universität München, James-Franck-Straße 1, DE-85748 Garching, Germany
#Physics Department and Institute for Advanced Study, Walter Schottky Institute, Technische Universität München, Am Coulombwall 4a, DE-85748 Garching, Germany

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ABSTRACT. Sub-unit vaccines typically show insufficient immunogenicity. To address this issue, we developed a novel self-adjuvanting particulate carrier system based upon the lipids phytantriol (Phy) and mannide monooleate (MaMo). Phy is a lipid known to form non-lamellar phases in fully hydrated systems, whereas MaMo has been found to promote immune responses in emulsion form. A bulk phase composition of Phy/MaMo (14 wt%) showed hexagonal (HII)
phase behavior over a practical temperature range (including room and body temperature), and was therefore used for particle development. Hexosomes stabilized with different concentrations of either poloxamer 407, Myrj 59 or Pluronic F108 were successfully prepared. To demonstrate the versatile nature of these systems, the particles were further modified with positively and negatively charged lipids and loaded with model antigens, whilst maintaining the HII structure. These hexosomes are structurally robust and amenable to customization, rendering them suitable as antigen delivery carriers.

**INTRODUCTION**

Vaccines are of utmost importance for global health and demonstrate one of the greatest medical successes. Especially vaccines based on highly purified and well-defined antigenic motifs (sub-unit vaccines) improved the safety and tolerability over the traditional whole-pathogen preparations.\(^1\) Nevertheless, sub-unit vaccines are often inefficient, because they are not able to induce the same level of immunogenicity demonstrated by the traditional ones.\(^2\) To circumvent these issues, several strategies have been considered. For instance, potent immunostimulatory compounds (adjuvants) are co-administered with the antigen molecules to improve the strength and type of the immune response.\(^3\) The incorporation of antigens into particulate delivery systems can prevent the premature degradation of the antigen *in-vivo* and at the same time allows mimicking the size, geometry, and kinetics of viruses and bacteria.\(^2\) Moreover, the particle design offers a range of possibilities for tuning interactions between antigen molecules and immune cells as well as overcoming multiple dose regimens.\(^2\) Progresses in the field of particulate delivery systems revealed promising potential for lipid-based particles such as cubosomes and hexosomes.\(^4\)\(^5\) Cubosomes are internally structured with an inverse bicontinuous cubic phase. They are formed by a highly twisted continuous lipid bilayer creating
a complex network with 3-D cubic symmetry and two non-intersecting water channels.\textsuperscript{6} Hexosomes are rod-shaped inverse micelles organized in a hexagonal structure with closed water channels.\textsuperscript{6} Due to their high interfacial areas and network of water channels, these particles are known as multi-compartment carriers and have great potential to overcome encapsulation constraints encountered with other particulate systems (liposomes, virus-like particles, and ISCOMs).\textsuperscript{4,7-8} Moreover, it has already been reported that non-lamellar structures (like cubic and hexagonal) show fusogenic properties,\textsuperscript{9-10} which could contribute to the delivery of antigens directly to the cytosol of antigen-presenting cells (APCs), stimulating cytotoxic T lymphocyte (CTL) immune responses.

The present work proposes the development of new self-assembled lipid-based particles with non-lamellar internal structures and immunostimulatory properties for vaccine delivery. For this purpose, the phase behavior of systems containing phytantriol (Phy) and mannide monooleate (MaMo) was investigated. Phy is a well-characterized lipid able to form non-lamellar phases in fully hydrated systems.\textsuperscript{11} MaMo is an emulsifier applied in several adjuvant systems (Montanide ISA 51 and 720, and Freund’s adjuvant)\textsuperscript{12} and it is known to promote Th1 and Th2 responses in emulsion form.\textsuperscript{13} The current approach aimed to synergistically combine the structural properties of Phy with the intrinsic biological activity of MaMo in one unique system to deliver antigens for immunization purposes. Different analytical methods, such as small-angle X-ray scattering (SAXS), cryogenic transmission electron microscopy (cryo-TEM) and polarization microscopy, were applied to characterize these new systems and to systematically develop customized nanostructured particles. The hexosomes could be modified with other charged lipids and model antigens without compromising the internal hexagonal structure. Taken together, the results
presented here emphasize the versatility of these systems and demonstrate their great application potential for the delivery of biopharmaceuticals.

**EXPERIMENTAL SECTION**

**Materials.** Phytantriol (Phy) from DSM Nutritional Products Europe Ltd was kindly provided by Nordmann, Rassmann GmbH (Hamburg, Germany). Mannide monooleate from plant (MaMo), dimethyldioctadecyl-ammonium bromide (DDA), poloxamer 407, albumin from chicken egg white (OVA), and lysozyme from chicken egg white (LYS) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Pluronic F108 and Myrj 59 were a gift from Croda (Barcelona, Spain). 1,2-dioleoyloxy-3-trimethylammonium propane chloride (Dotap), and 1,2-distearoyl-sn-glycero-3-phospho-rac-glycerol sodium salt (DSPG) were purchased from Lipoid GmbH (Ludwigshafen, Germany). CBQCA Protein Quantitation Kit was purchased from Life Technologies GmbH (Darmstadt, Germany). Phosphate buffer saline solution (PBS) was prepared at pH 7.2-7.6 using potassium chloride (Merck KGaA), potassium phosphate monobasic (Merck KGaA), sodium chloride (Merck KGaA), and sodium phosphate dibasic anhydrous (Grüssing GmbH).

**Preparation of Bulk Phases.** Appropriate amounts of Phy and MaMo were melted together at 70°C using the Thermomixer comfort (Eppendorf). In order to obtain a homogeneous molten, the samples were frequently vortexed. Highly purified water was added to obtain fully hydrated bulks (final lipid concentration of 50 wt%).

**Preparation of Particles.** Phy (86 mg), and MaMo (14 mg) were weighed and homogenized together as described for the bulk phases. To disperse particles from the bulk phase poloxamer 407, Pluronic F108 or Myrj 59 were applied as stabilizers in concentrations ranging from 5 to 30 wt% of the total lipid mass. The stabilizer solution in PBS was added to the Phy/MaMo mixture.
and followed by intense vortexing to give a final lipid concentration of 10 wt%. The samples were then agitated for 30 min at 3500 rpm in a dual asymmetric centrifuge (SpeedMixer\textsuperscript{TM}), followed by 3 cycles of sonication of 1 min with 4 s pulse, 2 s pause, and 20% amplitude (Bandelin Sonopuls HD3200 with sonotrode MS72). To introduce different particle surface charges, Phy and MaMo were homogenized with different amounts of charged lipids (DDA, Dotap, or DSPG). The samples were then processed as described above. In order to prepare protein loaded particles, appropriate amounts of either LYS or OVA were dissolved in the stabilizer solution and added to the lipid molten. Subsequently, the samples were agitated and sonicated as specified above. All samples were stored at room temperature (RT).

**Polarization Microscopy.** Polarization microscopy was performed as a preliminary screening method to select the bulk phases for SAXS measurements. The analyses were carried out using the Keyence microscope BZ-8100E equipped with a heating stage (Thermo Plate from TokaiHit) and polarization filters. A thin layer of sample (fully hydrated bulk) was applied between 2 cover glasses and observed under polarized light at temperatures from 25°C to 50°C. The presence of birefringence is an indication of lamellar (L\textalpha) or inverse hexagonal (HII) phases. Inverse bicontinuous cubic (QII) and micellar (L2) phases typically show isotropic behavior.\textsuperscript{14} Liposomes, hexosomes, cubosomes, and micelles can be derived from the dispersion of bulk phases with L\textalpha, HII, QII, and L2 structures, respectively.\textsuperscript{9}

**Small-Angle X-ray Scattering (SAXS).** SAXS analyses were accomplished using a Ganesha 300XL instrument equipped with a GENIX 3D microfocus Cu X-ray source and a 2D Pilatus 300K detector in a fully evacuated sample chamber. The bulk samples were mounted in a Linkam cell between mica windows (thickness 5-7 µm). For the characterization of particles, the dispersions were introduced into glass capillaries (1 mm light path), which were then
hermetically sealed and mounted in a heatable/coolable cell. The samples were measured after 10 min of equilibration at the selected temperature. The wavelength was $\lambda=1.54 \, \text{Å}$ and the sample-to-detector distance 401.4 mm (Linkam cell) and 406.2 mm (capillaries). The acquisition time was 30 and 120 min for bulks and particles, respectively. Longer acquisition times were used for the particles in order to increase the statistics and compensate the lower scattering signal. A pin diode was used to measure the transmission of the sample. The 2D images were azimuthally averaged and the background from the mica windows was subtracted, taking the transmission into account.

In order to identify the different liquid crystalline phases, the $q$-position ($q = 4\pi \sin(\theta/2)/\lambda$ is the momentum transfer) of each Bragg reflection was determined by fitting a Lorentz function. The ratios of the relative peak positions were calculated and compared to patterns described in the literature.\textsuperscript{15-16} The repeat distance $d$ was calculated using $d = 2\pi/q_1$, where $q_1$ is the position of the first peak. The mean lattice parameter $a$ was determined according to the scattering law for the respective mesophase, namely $a = \sqrt{2d}$ for Pn3m (cubic double diamond) and $a = 2d/\sqrt{3}$ for HII (inverse hexagonal).\textsuperscript{17}

**Dynamic Light Scattering (DLS).** Size and zeta potential analyses were performed using the Malvern Zetasizer Nano ZS (Malvern Instruments GmbH). Samples were diluted (1:200) and measured in PMMA cuvettes or DTS1060C capillary cells for the determination of size and zeta potential, respectively. All measurements were performed in triplicate, at 25°C, and in buffering conditions relevant for biological experiments (PBS, pH 7.4).

**Protein Load Determination.** In order to separate the free protein, the particles were washed four times with PBS using a centrifugal concentrator (Vivaspin 20 with PES membrane and MWCO 100k). To disrupt the particles and release potentially encapsulated protein molecules,
the washed samples were incubated for 30 min at RT with Triton-X 0.5%. After centrifugation (21000 g, 10 min), the protein content was quantified with the CBQCA Kit according to the manufacturer's protocol. Considering possible material loss that may occur during the washing steps, the values obtained after protein quantification were corrected by the dry mass of each sample (determined gravimetrically). The experiments were conducted in triplicates. Relative loading efficiencies were compared using a paired Student’s t-test performed by SigmaPlot 12.5 (Systat Software Inc., Germany).

**Tryptophan Accessibility Assay.** The accessibility of tryptophan residues to the collisional quencher acrylamide was evaluated. The fluorescence of protein molecules in buffered solution, and when loaded in particles, was investigated upon the addition of acrylamide at different concentrations (0.2 to 2 M). The fluorescence was monitored using the Cary Eclipse Fluorimeter at 25°C with excitation 280 nm (slit width 10 nm) and emission 300-400 nm (slit width 5 nm).

To compare the fluorescence intensities in different environments, the ratios between unquenched ($F_0$) and quenched ($F$) signals were calculated for each sample.

**Cryogenic Transmission Electron Microscopy (Cryo-TEM).** Vitrified specimens of selected formulations at approximately 5 wt% (total lipid) were prepared in a Vitrobot (FEI, The Netherlands) at 22°C and 90% humidity. A volume of 3 µl of the particle dispersion was applied on 400-mesh Quantifoil R2/1 copper grids (Quantifoil, Germany) that were negatively glow discharged with a Plasma Cleaner (EMS, USA) at 35 mA for 45 s. After 15 s equilibration, the excess of liquid was removed through blotting (3 s, blot force -1) and the grids were automatically vitrified in liquid ethane below -172°C. The samples were imaged using a Tecnai Spirit microscope (FEI, The Netherlands), equipped with an Eagle 4k charge-couple device (CCD) camera and a Cryo-Transfer Holder (Gatan, Inc., USA). The system was operated at an
acceleration voltage of 120 kV under low dose conditions (SerialEM acquisition software) with 26’000-, 30’000-, and 40’000-fold magnification. Image analysis was performed using Fiji.

RESULTS AND DISCUSSION

The design of novel particulate systems with non-lamellar internal structures and desired characteristics can be challenging, especially in complex formulations. Moreover, as it is very difficult to predict the impact of each additive on the mesophase structure, comprehensive studies are required. In the present work, formulations based on a combination of Phy and MaMo were systematically developed and the effects of various additives on relevant properties such as size, morphology, stability, charge, and loading were evaluated.

Characterization of Bulk Phases. To investigate the behavior of systems formed by Phy and MaMo, fully hydrated bulk phases of Phy/MaMo were prepared at different ratios in highly purified water and characterized using polarization microscopy and SAXS. Polarization microscopy was used to pre-screen a large number of samples and establish a phase diagram outline (Figure 1A), which enabled the selection of feasible temperature and concentration ranges for the SAXS analyses. The observation of anisotropy under polarized light is an indication of lamellar (Lα), or inverse hexagonal (HII) structures, which can yield liposomes or hexosomes, respectively, in dispersion. Isotropy, in turn, is an evidence of inverse bicontinuous cubic (QII), or micellar (L2) structures, which can be dispersed to give cubosomes or micelles, respectively.9,14 The analysis of liquid samples is challenging due to their high mobility, which hampered the visualization of transitions and birefringence. For this reason, the phase diagram outline was restricted to samples containing 0-20 wt% MaMo which were gel-like at RT.

As displayed in Figure 1A, bulks prepared with Phy at 100% appeared isotropic under polarized light at almost all temperatures investigated. This, together with the gel-like consistency,
illustrated the typical aspect of inverse bicontinuous cubic phases (QII). Likewise, Dong et al. observed isotropic properties and high viscosity for fully hydrated bulks of Phy. However, they reported a transition to anisotropic behavior at temperatures above 60°C, while in the present work, the formation of birefringent structures occurred already at 50°C (Figure 1A). The phase diagram outline (Figure 1A) revealed that even low concentrations of MaMo had a remarkable impact on the structure of pure Phy. At 2-4 wt%, MaMo induced a transition from iso- to anisotropic behavior with an intermediate region showing characteristics of both phases. This transition can be seen in more detail in Figure 1B, in which the micrographs obtained for a fully hydrated bulk formed by 3.3 wt% MaMo in Phy are displayed. At 25°C, the isotropic and featureless aspect of this bulk was evident. In addition, the high viscosity of the sample was noticeable through its irregular interface, which suggested an inverse cubic structure (QII). At 33°C, the formation of small birefringent areas was observed. This anisotropic aspect is characteristic for both, lamellar (Lα), and inverse hexagonal (HII) phases. At higher temperatures (34-50°C), the anisotropic properties became gradually more dominant and the addition of more MaMo (Figure 1A) shifted the transition from iso- to anisotropic behavior to temperatures below 25°C.

These findings demonstrated the remarkable ability of MaMo to modify the phase behavior of Phy and indicated the establishment of an anisotropic mesophase at room temperature (Figure 1A). At 15-20 wt% MaMo, the bulk samples underwent a transition from anisotropic to isotropic at higher temperatures (above 35°C). In comparison with the initial isotropic structure, the second isotropic phase displayed higher fluidity, which may be an indication of a micellar (L2) structure.
In order to fully understand the impact of MaMo on Phy systems and to precisely identify the mesophases along with their internal arrangements in a broader concentration and temperature range, SAXS measurements were performed. As shown in Figure 2A, in pure Phy systems, a phase transition from inverse bicontinuous cubic double diamond (Pn3m) to HII occurred at approximately 50°C. At 60°C, a pure HII topology was identified. Interestingly, this structure did not prevail in a large temperature range, and already at 70°C, the sample displayed an inverse micellar (L2) structure. Similarly, the characterization of Phy bulks reported by other groups\textsuperscript{18, 20} revealed Pn3m structures at room temperature, followed by transitions to HII and subsequently to L2 upon temperature increase. However, the literature is not fully consistent with respect to the phase transition temperatures determined in the present work. Deviations of up to 10°C were identified.\textsuperscript{7} It is known that the origin of the substances used plays a decisive role in this matter, i.e. the phase behavior strongly depends on the purity of the Phy used.\textsuperscript{7, 21} This may explain the deviations from reports of other groups.

Higher MaMo ratios facilitated the formation of HII structures (Figure 2A). The phase transition from Pn3m to HII observed in pure Phy systems at 50°C was reduced to values below 25°C. Analogously, the temperature of the phase transition from HII to L2 was progressively decreased as the MaMo content increased (from 60-70°C in 100% Phy to 25-37°C in 28 wt% MaMo in Phy). Samples containing 40-80 wt% MaMo in Phy showed phase separation, while concentrations above 80 wt% resulted in stable and homogeneous samples with L2 structure.

The phase modulation demonstrated by MaMo in Phy bulks is comparable to other lipidic systems, such as Phy/vitamin E acetate,\textsuperscript{18} glyceryl monooleate/diolein,\textsuperscript{22} and glyceryl monooleate/oleic acid.\textsuperscript{23} Analogously, the original inverse cubic structure was successfully
transformed to HII with the incorporation of each component in a concentration dependent fashion.\textsuperscript{18, 22-23}

Among all Phy/MaMo ratios evaluated, Phy bulk phases containing 14 wt\% MaMo were found to be the most suitable for this study. At this MaMo content, HII mesophases were formed and maintained over the largest temperature range, including body and room temperature, as indicated by SAXS analysis (Figure 2B). Interestingly, the SAXS results revealed that a temperature increase from 25 to 37°C promoted a reduction of the lattice parameter from 4.85 ± 0.01 to 4.63 ± 0.01 nm. This temperature dependent behavior was seen in all samples and may be explained by the higher conformational disorder and volume expansion in the chain region of the lipid molecules at higher temperatures.\textsuperscript{9} At 50°C, the first and most intense peak became broader, while the intensity of the second and third peak was reduced, indicating a HII-L2 phase transition (Figure 2B). At 60°C and 70°C, the typical scattering profile for L2 structures, featuring only one broad peak, was observed. The presence of HII structures over a large temperature range may be advantageous for the customization of the systems and particle stabilization without sacrificing the non-lamellar profile.

**Particle Preparation.** The preparation of particles requires the addition of a stabilizing agent, which enables the dispersion of the bulk phase and prevents particle aggregation and/or flocculation.\textsuperscript{7} However, depending on the concentration used, this emulsifier can increase the toxicity of the particles and also disturb their internal structure.\textsuperscript{24} Poloxamer 407 (also known as Pluronic F127) is the most commonly used stabilizing agent in lyotropic liquid dispersions.\textsuperscript{7} A recent study identified Pluronic F108 (poloxamer 338) to be even more effective as a stabilizer than Pluronic F127 for dispersed systems composed of Phy or glyceryl monooleate.\textsuperscript{25} Similarly,
an excellent stabilization capacity of Phy-based cubosomes has also been reported for Myrj 59 (Polyoxyethylene-100-stearate).\textsuperscript{26}

The feasibility of these three stabilizers to disperse concentrated Phy/MaMo systems and to yield hexosomes within the submicron range was evaluated in the present study. The results obtained for the size analyses of the particles through dynamic light scattering (DLS) are depicted in Figure 3 and indicated that all stabilizers effectively dispersed the Phy/MaMo bulks. SAXS analyses of the samples containing 5 and 30 wt\% of each stabilizer showed that the internal inverse hexagonal structure of the particles was maintained (Figure S1). As displayed in Figure 3, at high concentrations (above 20 wt\%), all stabilizers promoted a drastic reduction in size and polydispersity of the samples. Such remarkable impact of stabilizer concentration on the particle size profile has not been observed in similar investigations.\textsuperscript{18, 25-26} An explanation may be that the size is not only determined by the stabilizer type and concentration, but also by the dispersion method. Dong et al. prepared Phy/vitamin E acetate hexosomes stabilized with 10 wt\% Pluronic F127 and reported a diameter of approximately 250 nm and polydispersity index (PdI) below 0.25.\textsuperscript{18} These results strongly contrasted with the sizes displayed in Figure 3A, which can be attributed to the different ultrasonication protocols used. In the present study, the hexosomes were prepared using mild conditions (3 cycles: 1 min of 4 s pulse/2 s pause, 20\% amplitude), while Dong et al.\textsuperscript{18} applied much higher energy input (20 min of 0.5 s pulse/0.5 s pause, 40\% amplitude). We assumed that high-energy dispersion methods are able to increase the efficiency of the stabilizing agent, diminishing the concentration dependence displayed in Figure 3. This was supported by the preparation of Phy/MaMo hexosomes stabilized with 5 wt\% poloxamer 407 using harsh ultrasonication conditions (2 cycles: 5 min of 4 s pulse/2 s pause, 50\% amplitude) that yielded particles with an approximate diameter of 200 nm and a PdI below
0.15. Despite the enhancement of the size profile, the energy excess is mainly dissipated as heat, which makes these conditions unsuitable for potential encapsulation of proteinaceous material.

Based on the reduction of the particle size seen in Figure 3, it may be assumed that at 5 wt% Myrj 59 dispersed the bulk phase more efficiently than the other stabilizers. However, when considering the number of stabilizer molecules necessary to stabilize a specific number of lipid molecules, 5 wt% of Myrj 59, corresponding to 0.37 mol%, can be practically compared to samples stabilized with 15 wt% poloxamer 407 and Pluronic F108, corresponding to 0.41 and 0.35 mol%, respectively, within this data set. This comparison revealed that Myrj 59 is the least potent stabilizing agent and thus contradicts descriptions by Chong et al., in which Myrj 59 was considered a more potent stabilizing agent than Pluronic F127.\textsuperscript{26} Particles dispersed with Pluronic F108 and poloxamer 407 showed comparable size profiles, whereby Pluronic F108 promoted a slightly better size reduction, possibly due to its longer polyoxypropylene chain.\textsuperscript{25}

It is important to consider that stabilizers with longer chains may promote a more intensive stealth effect in biological systems, as observed with a standard PEGylation. In this work, the hexosomes are intended to be used for vaccine delivery, and the particle uptake by the immune cells is a desired effect. For this reason, stabilizing agents with shorter molecules, such as poloxamer 407 and Myrj 59, are preferred over Pluronic F108.

**Particle Size Optimization.** The formation of non-lamellar structured particles is typically achieved through the dispersion of the bulk phase in the presence of stabilizers.\textsuperscript{14,27} In order to prepare relatively monodisperse systems with stable particles in the submicron range, the dispersion technique is followed by a post-treatment to further improve the size profile. For instance, heat treatment (125°C for 20 min)\textsuperscript{28}, autoclaving (121°C for 15 min at 1 bar)\textsuperscript{29} and high pressure homogenization\textsuperscript{30} have been reported as suitable dispersion post-treatment methods to
reduce the size distribution of glyceryl monooleate cubosomes stabilized with Pluronic F127. Figure 4 shows strategies applied in the present work to optimize the size profile of Phy/MaMo particles stabilized with 30 wt% Myrj 59. When incubated overnight at 40°C, the particles showed a drastic size reduction in comparison to samples kept at room temperature (Figure 4A). Maintaining constant storage conditions, size and Pdi remained constant over 45 days. Interestingly, samples not subjected to this temperature treatment underwent a progressive size reduction over time.

These results suggested that the temperature treatment accelerated an equilibration process of the system, yielding a stable and relatively monodisperse particle dispersion that can be stored at RT for at least 45 days. This phenomenon was observed only in samples stabilized with Myrj 59, and to date, to the best of our knowledge, there are no reports addressing similar findings. Studies on cubosomes stabilized with Pluronic F127 showed that intensive heat treatment (125°C for 20 min) yielded larger particles with a more narrow size distribution, while lower temperatures for longer time periods (95°C for two hours) did not induce the same effects. It may be hypothesized that Pluronic F127 and Myrj 59 have different stabilization mechanisms and interact differently with the lipidic structure.

Alternatively, three-fold extrusion of the formulations through a polycarbonate membrane of 400 nm pore size yielded stable particles with a similar size profile (Figure 4B). One major advantage of the temperature treatment over the extrusion is that it maintains the sample integrity, because material loss is not possible. However, depending on the encapsulated content, the temperature treatment may induce instability issues and premature release. SAXS analysis confirmed the conservation of the HII structures after both size optimization strategies (Figure 4C) and affirmed that both methods are suitable to improve the size profile of these systems.
**Particle Customization.** Considering the relevance of the overall surface charge of delivery systems in the interactions with cells, hexosomes carrying a positive or negative overall surface charge were developed in the present work. For this purpose, DDA (+), Dotap (+), and DSPG (-) were added at various concentrations (5-30 wt%) to the standard Phy/MaMo (14 wt%) formulation. The measured zeta potential of each hexosome formulation correlated with the amount of incorporated charged lipid, whereby increased concentrations resulted in higher absolute values (positive/negative) (Figure 5).

The nature of the charged lipid and its concentration are critical for the internal structure of the particles.\(^7\) SAXS was used to verify whether the hexagonal structure in samples containing relatively high concentrations of charged lipids was maintained (Figure 6). The addition of DDA had a considerable impact on the HII mesophase at 25°C (Figure 6). The first and third peak at 1.217±0.003 and 1.481±0.006 nm\(^{-1}\), respectively, corresponded to the \(\sqrt{2}\) and \(\sqrt{3}\) reflections and indicated Pn3m structures. This suggested a transition or coexistence of Pn3m and HII phases. Within the lipid domain, the positive charge of DDA increased the repulsion between the headgroups. It was assumed that this imbalance of lateral stresses reduced the negative curvature of the structure, which promoted a transition from a hexagonal to a cubic geometry.\(^{32-33}\)

Interestingly, a temperature increase from 25°C to 37°C promoted a recovery of the system, which displayed peaks at 1.381±0.001, 2.395±0.006, and 2.752±0.009 nm\(^{-1}\) (corresponding to 1, \(\sqrt{3}\), and \(\sqrt{4}\) reflections in Figure 6) and represented a predominant HII profile. A plausible explanation may be that higher temperature led to greater conformational disorder and splay in the chain region and so compensated the lateral stresses between the headgroups.\(^9\) The HII structure formed at 37°C showed Bragg reflections at lower \(q\) values than the original bulks (black curves) suggesting an enlargement of the cell unit.
Formulations containing Dotap (blue curves), and DSPG (red curves) maintained the HII structure at both temperatures and showed an increased lattice parameter from 4.84±0.01 nm (bulk phase) to 5.62±0.01 and 5.75±0.01 nm for Dotap and DSPG, respectively (at 25°C, Table 1). A higher lattice parameter correlates with an enlargement of the water channel diameter. This modulation of the internal structure may be used as potential tool for drug encapsulation.

Similarly to the Phy/MaMo bulk phases (Figure 2B), hexosomes containing DSPG and Dotap revealed a reduction of the lattice parameter upon temperature increase (Table 1). However, this behavior was less pronounced, which is most likely due to repulsion between the charged headgroups and sterical hindrance.

The effect of DDA on the internal structure in comparison to Dotap at similar molar concentrations was surprising (Figure S2). Due to its smaller headgroup and saturated hydrocarbon chains, DDA was expected to show minimal impact on the hexagonal structure. However, it seemed to induce a transition from the hexagonal to the cubic phase, whereas Dotap, which consists of a trimethylammonium headgroup and two unsaturated hydrocarbon chains, only enlarged the lattice parameter. As mentioned earlier, it is believed that due to its molecular structure, DDA generated an imbalance between the hydrophobic chains and headgroup. Even though Dotap is a more voluminous molecule than DDA, the results indicated that the headgroup and hydrocarbon chain regions contributed more equally to the packing parameter and the HII structure was preserved.

The effect of the addition of charged lipids on the structure of Phy/MaMo hexosomes was also observed with Cryo-TEM (Figure 7). The electron micrographs of the standard formulation (without addition of charged lipid), and formulations containing 25 wt% Dotap or 15 wt% DSPG revealed particles with clear parallel lines forming curved striations, and/or hexagonal shape,
which are characteristic for hexosomes.\textsuperscript{35} Differently, samples containing 30 wt\% DDA displayed a more undefined structural profile. The particles appeared to have a spherical shape and showed internal structures with undulated distorted lines. These observations are in agreement with the SAXS measurements (Figure 7).

**Particle Loading.** The entrapment of two different model antigens, namely lysozyme (LYS) and ovalbumin (OVA), into the delivery systems and its impact on the HII structure of Phy/MaMo (14 wt\%) was investigated. In comparison to OVA, LYS is smaller in size and positively charged at pH 7.4 (Table 2). Due to these characteristics, a simple encapsulation and interactions with the negatively charged Phy/MaMo hexosomes were thought to be facilitated for LYS, which could result in a higher loading efficiency in comparison to OVA. The macromolecules were added to the homogenized lipid molten in a buffered solution together with the stabilizer. According to the literature, lipid molecules self-assemble upon contact with aqueous medium containing a model antigen, which is then enclosed within the water channels.\textsuperscript{5, 36} Contrary to the expectations, the relative loading efficiency obtained for OVA was significantly higher compared to LYS (rLE\% of 25 vs. 7, $p \leq 0.001$, Table 2).

SAXS measurements of hexosomes loaded with either OVA or LYS revealed that the HII topology and lattice parameter were conserved for both proteins (Figure 8A). However, when comparing the dimensions of these proteins (Table 2) and the lattice parameter (distance between the centers of two adjacent cylinders)\textsuperscript{37} of 4.8 nm, a simple encapsulation into the water channels without a noticeable impact on the structure is highly unlikely. An alternative loading mechanism suggested by Angelova et al.\textsuperscript{38} proposes the association of protein molecules to the interfaces of the domains of cubic arrangement within the cubosomes, creating "pockets of local disorder".\textsuperscript{39} This mechanism may explain the considerable rLE\%, despite the geometric
mismatch between the proteins and water channels, but it does not justify the absence of modifications in the Phy/MaMo HII structure.

To obtain insights into the localization of the loaded protein within the hexosomes, a tryptophan accessibility assay was performed, whereby the fluorescence of tryptophan residues is quenched with acrylamide. Proteins encapsulated within the hexosomes are in a more isolated environment and thus less exposed to the acrylamide than proteins adsorbed to the particle surface.\(^{40}\) In contrast to the buffer solution, less quenching of the fluorescence and thus lower quencher efficiencies \((F_0/F)\) was observed for both proteins when loaded into hexosomal formulation (Figure S3). This suggested that OVA and LYS were to some extent integrated into the lipidic structure. However, in direct comparison to OVA, LYS showed a stronger reduction of the quenching efficiency when it was loaded into hexosomes. This indicated that the incorporation into hexosomes shielded LYS from the aqueous environment more efficiently than OVA and thereby, reduced its availability for acrylamide quenching.

The Phy/MaMo (14 wt\%) system was further challenged by simultaneously including 30 wt\% DDA and either OVA or LYS as a model antigen (Figure 8B). As discussed earlier, the inclusion of DDA led to a combination of cubic and hexagonal phases. Upon protein loading, the intensity of the Pn3m reflections, at approximately 1.22 and 1.48 nm\(^{-1}\), were reduced (indicated by stars in Figure B). Additionally, the reflections observed at \(q > 2.0\) nm\(^{-1}\) appeared more visible for loaded particles. Overall, the protein addition seemed to drive the systems back to pure HII assemblies. This effect was more profound for samples containing LYS. This ability of both proteins to modify the internal structure suggested a strong association of the protein molecules with the lipid network.
A comparable recovery of the HII structure was seen for plain hexosomes containing 30 wt% DDA (Figure 6) upon temperature increase. With rising temperature, the fluidity of the hydrocarbon chains increased, and compensated the lateral stresses introduced by the positive headgroups of DDA. Similarly to the temperature effect, the incorporation of protein molecules may have caused a volume expansion in the hydrophobic chain region, which contributed to the reestablishment of the hexagonal structure. These findings suggested that the proteins mostly interacted with the hydrophobic chains via nonpolar interactions. That this effect was more pronounced for LYS is in agreement with findings from the tryptophan accessibility assay. Considering that both, the lipid assembly and LYS, were positively charged at pH 7.4 (Table 2), it may be hypothesized that the loading mechanism is mainly driven by the hydrophobic forces rather than electrostatic interactions.

**CONCLUSION AND OUTLOOK**

This study demonstrated the rational development of novel vaccine delivery systems based on internally structured particles. Phy/MaMo (14 wt%) hexosomes were successfully dispersed from their bulk phases using either poloxamer 407, Myrj 59, or Pluronic F108. The particle size profiles of the various formulations were a result of the combination of dispersion method, as well as the type and concentration of the stabilizer. Different approaches, such as inclusion of various additives and dispersion post-treatments, were combined in order to obtain systems with desired characteristics. Phy/MaMo (14 wt%) revealed broad margins to tune relevant properties without sacrificing the HII internal arrangement. For example, the incorporation of charged lipids to modify the overall particle charge, as well as the loading of different model proteins could be performed without compromising the HII profile of the particles. This remarkable structural robustness is unusual in lyotropic liquid crystalline dispersions, which are known for
their susceptibility to phase transformations without satisfactory predictability. Future efforts are needed to fully understand the role of internally structured particles in living systems. In this context, relevant investigations will be performed to further optimize hexosomes and improve their current status as antigen delivery system.

ASSOCIATED CONTENT

Supporting Information. Figures S1-S3 are supplied as Supporting Information. Figure S1 provides additional SAXS results regarding the effect of different concentrations of steric stabilizers (poloxamer 407, Myrj 59, and Pluronic F108) on the HII internal structure of Phy/MaMo (14 wt%) hexosomes. Figure S2 displays SAXS curves for Phy/MaMo (14 wt%) formulations containing the same molar concentration of the charged lipids, Dotap and DDA. Figure S3 shows the results of the tryptophan accessibility assay performed with protein loaded hexosomes. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Department of Integrative Medical Biology, Umeå University, 901 87 Umeå, Sweden, E-mail: madlen.hubert@umu.se, Phone: +46 73 6737781, Fax: +46 90 786 0000.

Present Addresses

§Department of Integrative Medical Biology, Umeå University, 901 87 Umeå, Sweden.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS
Cryo-TEM, cryogenic transmission electron microscopy; DDA, dimethyldioctadecyl-ammonium bromide; DLS, dynamic light scattering; Dotap, 1,2-dioleoyloxy-3-trimethylammonium propane chloride; DSPG, 1,2-distearoyl-sn-glycero-3-phospho-rac-glycerol sodium salt; LYS, lysozyme from chicken egg white; MaMo, mannide monooleate; OVA, albumin from chicken egg white; Phy, phytantriol; PBS, phosphate buffer saline solution; rLE%, relative loading efficiency; RT, room temperature; SAXS, small-angle X-ray scattering; SD, standard deviation; wt, weight.
Table 1. Change in lattice parameter upon temperature increase of charged Phy/MaMo (14 wt%) hexosomes stabilized with 10 wt% Poloxamer 407.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lattice Parameter</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>at 25°C (nm)</td>
<td>at 37°C (nm)</td>
<td>Difference** (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bulk*</td>
<td>4.84</td>
<td>4.64</td>
<td>4.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexosomes + 25 wt% Dotap</td>
<td>5.62</td>
<td>5.55</td>
<td>1.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexosomes + 15 wt% DSPG</td>
<td>5.75</td>
<td>5.66</td>
<td>1.61</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*bulks do not contain stabilizers and charged lipids. **lattice parameter reduction from 25°C to 37°C.

Table 2. Characteristics of the model proteins used in the loading studies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Dimensions (nm)</th>
<th>pI*</th>
<th>Charge at pH 7.4</th>
<th>rLE% ± SD**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (14.2 kDa)</td>
<td>2.8 x 3.2 x 3.0</td>
<td>11</td>
<td>Positive</td>
<td>7 ± 3</td>
</tr>
<tr>
<td>Ovalbumin (42.7 kDa)</td>
<td>7.0 x 3.6 x 3.0</td>
<td>4.5</td>
<td>Negative</td>
<td>25 ± 3</td>
</tr>
</tbody>
</table>

*pI (Isoelectric point); **rLE% ± SD (Mean relative loading efficiency ± standard deviation).
Figure 1. A: Phase diagram outline established through polarization microscopy of fully hydrated bulk phases formed by Phy and MaMo at different weight ratios and temperatures. B: Polarization micrographs of a fully hydrated bulk formed by 3.3 wt% of MaMo in Phy at different temperatures.
Figure 2. A: Phase diagram obtained by SAXS measurements of fully-hydrated bulk phases formed by Phy and MaMo at different weight ratios and temperatures. B: SAXS curves as a function of temperature of 14 wt% MaMo in Phy (fully hydrated bulk phase); Pn3m (inverse bicontinuous cubic double diamond); HII (inverse hexagonal); L2 (inverse micellar).
Figure 3. Size characterization by dynamic light scattering of Phy/MaMo (14 wt%) formulations containing poloxamer 407 (A), Pluronic F108 (B), and Myrj 59 (C) at different concentrations. The stabilizer concentrations are given in weight% and in mol% of the total lipid in the formulation. Bars and dots depict mean ± SD of three independent measurements.
Figure 4. Size optimization strategies shown for Phy/MaMo (14 wt%) hexosomes stabilized with 30 wt% Myrj 59. A: Size (columns) and PDI (squares) monitored by DLS of samples stored at room temperature (RT) (dark grey), and samples incubated overnight at 40°C (light grey). B: Effect of size optimization using temperature treatment at 40°C and extrusion through a polycarbonate membrane of 400 nm pore size. Data is displayed as mean ± SD of three independent measurements. C: SAXS curves from particles without size optimization (black), subjected to extrusion through a polycarbonate membrane of 400 nm pore size (red), and subjected to temperature treatment overnight at 40°C (blue).

Figure 5. Zeta potential values of Phy/MaMo (14 wt%) hexosomes stabilized with 15 wt% poloxamer 407 containing different amounts of charged lipids, as given in the graph in wt% of the total lipid amount. All measurements were performed at RT, in buffering conditions relevant for biological studies (PBS, pH 7.4). Data shows the average of three independent measurements ± SD.
Figure 6. Effect of additional charged lipid on Phy/MaMo (14 wt%) hexosomes stabilized with 15 wt% Poloxamer 407. SAXS patterns are shown for the following samples at 25°C and 37°C: standard Phy/MaMo (14 wt%) bulk phase (black), 30 wt% DDA (green), 25 wt% Dotap (blue), and 15 wt% DSPG (red). The curves were shifted vertically for clarity. The vertical dashed line highlights a shift of the Bragg reflections to lower q values for samples containing charged lipids in comparison with the original Phy/MaMo (14 wt%) bulk.
Figure 7. Cryo-TEM images of Phy/MaMo (14 wt%) formulations stabilized with 15 wt% Poloxamer 407. A: without additional charged lipid; B: addition of 30 wt% DDA; C: addition of 25 wt% Dotap; D: addition of 15 wt% DSPG.
Figure 8. A: SAXS curves of hexosomes formed by Phy/MaMo (14 wt%), stabilized with 5 wt% poloxamer 407 and loaded with either 1 wt% (of total lipid) of LYS (blue) or OVA (red). B: SAXS characterization of hexosomes formed by Phy/MaMo (14 wt%) and 30 wt% DDA, stabilized with 15 wt% poloxamer 407. Plain hexosomes (red), loaded with 1 wt% OVA (green), loaded with 1 wt% LYS (blue). Black curve represents the bulk phase (without stabilizer, DDA,
and protein) for comparison. Curves were shifted vertically for clarity. Measurements were performed at 25°C.
REFERENCES

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Table of Contents/Abstract Graphic:

Phytantriol

Protein Loading
Surface Charge
Customization
Stabilization

Pn3m

+ Mannide Monooleate

HII