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Citrem modulates internal nanostructure of glyceryl monooleate dispersions and bypasses complement activation: Towards development of safe tunable intravenous lipid nanocarriers

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Abstract

Lyotropic non-lamellar liquid crystalline (LLC) aqueous nanodispersions hold a great promise in drug solubilization and delivery, but these nanosystems often induce severe hemolysis and complement activation, which limit their applications for safe intravenous administration. Here, we engineer and characterize LLC aqueous nanodispersions from a binary lipid mixture consisting of 2,3-dihydroxypropyl oleate (glyceryl monooleate) and medium-chain triglyceride with tunable internal nanostructures and improved hemocompatibility controlled by citrem as stabilizer. Citrem, in a concentration-dependent manner, modulates the internal nanostructure of LLC dispersions from a biphasic H_2/L_2 feature to a neat L_2 phase, where the latter resembles "thread-like" swollen micelles. Citrem stabilization totally overcomes hemolysis and complement activation, thus realizing the potential of the engineered LLC aqueous nanodispersions for exploitation in intravenous delivery of drugs and contrast agents.

Key words: Citrem; Complement system; Hierarchical materials; Hexosomes; Synchrotron small-angle X-ray scattering (SAXS)

Lyotropic non-lamellar liquid crystalline (LLC) aqueous dispersions of cubosome and hexosome classes exhibit highly ordered and thermodynamically stable internal tunable nanostructures with attributes that make them promising candidates for solubilization of zwitterionic, charged and hydrophobic drugs.[1,2] However, compatibility of these lipid systems with elements of the human blood remains poor, and therefore in need of optimization.[3-5] For instance, the commonly used phytantriol (PT)-based lipid dispersions induce hemolysis.[3] Tri-block copolymers such as Pluronic® F127 as well as PEGylated and polyethoxylated fatty acids are widely used for stabilization of cubosomes and hexosomes,[5-9] but such stabilizers activate the human complement system in both micellar and surface immobilized forms.[10-12] The complement system is the first line of the body's defense against intruders, where its inadvertent activation may contribute to adverse reactions with cardiovascular, bronchopulmonary, mucocutaneous, neuropsychosomatic and autonomic manifestations.[12,13] Indeed, such reactions have been noted in some patients receiving intravenous infusion of

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Table 1
Composition and biophysical characteristics of the LLC aqueous nanodispersions.

LLC dispersion	GMO:MCT	Lipid content (wt%)	Stabilizer	Stabilizer content (wt%)	Medium[a]	Phase	*d* (nm)	*a* (nm)	Mean diameter[b] (nm)	Median diameter[b] (nm)
LD_{F127}	90:10	10	F127	3	Buffer	H_2	–	5.45	94 ± 8	82
					Serum	H_2	–	5.52	118 ± 22	101
$LD_{citrem1.5}$	85:15	5	Citrem	1.5	Buffer	H_2/L_2	–	5.82	147 ± 10	142
					Serum	H_2/L_2	–	6.09	–	–
$LD_{citrem3.0}$	85:15	5	Citrem	3	Buffer	L_2	5.40	–	119 ± 8	103
					Serum	L_2	6.47	–	127 ± 13	120

GMO: glyceryl monooleate; MCT: medium-chain triglycerides; *d*: characteristic distance; *a*: lattice parameter.
[a] For incubation conditions see the experimental section.
[b] Determined by Nanoparticle Tracking Analysis (NTA).

lipid- and polymer-based nanomedicines, including long-circulating formulations.[12,13] Here, we describe a simple strategy in LLC aqueous nanodispersion engineering with tunable internal nanostructures and improved hemocompatibility. These engineered nanostructures bypass hemolysis and do not incite complement, thus realizing their potential for exploitation in intravenous delivery of drugs and contrast agents.

Methods

Details for the preparation of lipid dispersions and their characterization by Nanoparticle Tracking Analysis, cryogenic transmission electron microscopy (cryo-TEM), and synchrotron small-angle X-ray scattering (SAXS) were reported before,[14] and presented in the supplementary material. Details for the hemolysis test and complement activation studies were in accordance with our previous studies[10,15] (Supplementary Material).

Results and Discussion

We used a binary lipid mixture consisting of 2,3-dihydroxypropyl oleate (glyceryl monooleate, GMO), which is the most investigated surfactant-like lipid with a non-lamellar propensity, and medium-chain triglyceride (MCT) to form LLC aqueous nanodispersions stabilized with citrem[14] (LD_{citrem}), Table 1. Citrem is an anionic citric acid ester of monoglycerides, which has been approved by the United States Food and Drug Administration as an emulsifying agent in food products.[14] For comparison, we prepared GMO/MCT nanodispersions stabilized with the most commonly used stabilizer Pluronic® F127 (LD_{F127}). The SAXS pattern for LD_{F127} showed three Bragg reflections ((10), (11) and (20)) characteristic for an internal inverted hexagonal (H_2) phase (hexosomes),[16] Figure 1, *A*, and this finding was supported by cryo-TEM (Figure 1, *D*). On the contrary, with a dispersion stabilized with 1.5 wt% citrem ($LD_{citrem1.5}$) there was an increase in the lattice parameter of the internal H_2 phase from 5.45 to 5.82 nm showing an additional broad peak at $q \sim 1\ nm^{-1}$ (Figure 1, *B*). This indicates co-existence of a less ordered internal inverted-type microemulsion (internal L_2 phase).[17] However, increasing citrem concentration to 3 wt% ($LD_{citrem3.0}$) transformed the internal biphasic H_2/L_2 structure to a neat L_2 phase (emulsified microemulsions, EMEs). The identification of this phase was based on the detection of a single broad peak at $q = 1.16\ nm^{-1}$. On the basis of cryo-TEM observations we suggest that the interior nanostructure of EME closely resembles "thread-like" swollen micelles (Figure 1, *D*). Citrem may incorporate into the hydrophobic domain of the internal GMO/MCT nanostructure as well as in the water/GMO interface due to its surface-active property. Accordingly, the aforementioned transition is most likely regulated by the extent of citrem incorporation into the hydrophobic GMO/MCT domains, thereby modulating the critical packing parameter, and consequently increasing the negative mean curvature.[14]

On serum incubation (4 h, 37 °C), the internal H_2 nanostructure of LD_{F127} was retained, but showed a slight increase in the lattice parameter of the H_2 phase (from 5.45 to 5.52 nm) (Figure 1, *C*, Table 1, Supplementary Figure S1). Both citrem-stabilized nanodispersions also showed swelling of the internal structure on serum incubation, as viewed by an increase in the lattice parameter (*a*) of the internal H_2 phase and the characteristic distance (*d*) of the internal L_2 phase (Figure 1, *C*, Table 1, Supplementary Figure S2). With $LD_{citrem3.0}$, the increase in *d* was dramatic (from 5.40 to 6.47 nm, corresponding to 19.8%), whereas with $LD_{citrem1.5}$ the lattice parameter of the internal H_2 phase was increased marginally (from 5.82 to 6.09 nm, corresponding to 4.6%). This notable enlargement of the L_2 phase may arise either from interaction of the interior nanostructure of GMO/MCT with serum components and/or trapping of serum proteins in the aqueous core of the "swollen" inverted type micelles (Figure 1, *E*). Accordingly, the microenvironmental conditions of the hydrophilic cylindrical nanochannels of the H_2 phase limits trapping of large amount of serum components, since only a slight serum-mediated enlargement of the hydrophilic pores is detectable.

Next, we compared membrane disruptive properties of LD_{F127} and $LD_{citrem3.0}$ by examining erythrocyte lysis in fresh human whole blood (WB) (Figure 2). We chose lepirudin as an anticoagulant (a specific thrombin inhibitor), which has no effect on membrane integrity and the complement system.[18] LD_{F127} showed concentration-dependent hemolysis in the WB with a significant membrane damage even at low lipid concentration (0.05 wt%). In the absence of plasma, hemolysis was considerably higher, starting at a lipid concentration of 0.01 wt%. In contrast, $LD_{citrem3.0}$ did not induce hemolysis in the WB even at a high lipid concentration (0.20 wt%), but hemolysis could proceed in the absence of plasma (a non-physiological condition)