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REVIEW

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# Structural characterization of lipidic systems under nonequilibrium conditions

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**Abstract** This review covers recent studies on the characterization of the dynamics of lipidic nanostructures formed via self-assembly processes. The focus is placed on two main topics: First, an overview of advanced experimental small-angle X-ray scattering (SAXS) setups combined with various sample manipulation techniques including, for instance, stop-flow mixing or rapid temperature-jump perturbation is given. Second, our recent synchrotron SAXS findings on the dynamic structural response of gold nanoparticle-loaded vesicles upon exposure to an ultraviolet light source, the impact of rapidly mixing negatively charged vesicles with calcium ions, and in situ hydration-induced formation of inverted-type liquid-crystalline phases loaded with the local anesthetic bupivacaine are summarized. These in situ time-resolved experiments allow real-time monitoring of the dynamics of the structural changes and the possible formation of intermediate states in the millisecond to second range. The need for investigating self-assembled systems, mainly stimuli-responsive drug nanocarriers, under nonequilibrium conditions is discussed. For pharmaceutically relevant applications, it is essential to combine these investigations with appropriate in vitro and in vivo studies.

Special Issue: Scattering techniques in biology—Marking the contributions to the field from Peter Laggner on the occasion of his 68th birthday.

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## Introduction

Attractive lipidic nanoscale systems for pharmaceutical applications including liposomal formulations and inverted-type liquid-crystalline phases and their aqueous dispersions (cubosomes and hexosomes) have attracted considerable attention in recent years (Moghimi et al. 2011; Preiss and Bothun 2011; Couvreur and Vauthier 2006; Paasonen et al. 2010; Yaghmur and Glatter 2009; Malmsten 2006; Yaghmur and Rappolt 2011; Angelova et al. 2005). In particular, there is growing interest in utilizing these systems as nanocarriers for anticancer drugs (Moghimi et al. 2011; Namiki et al. 2011; Couvreur and Vauthier 2006). A key challenge in the application of these nanoscaled, highly organized hierarchical assemblies as drug nanocarriers is achieving safe and biocompatible systems with high drug bioavailability and efficient cellular uptake and targeting (Moghimi et al. 2011; Preiss and Bothun 2011; Couvreur and Vauthier 2006). This important point has to be addressed by investigating the physicochemical properties of these formulations and performing relevant clinical studies. It requires also full understanding of their stability and dynamic behavior after administration. In this regard, it is important for various applications to learn the response of these lipidic self-assembled nanostructures to environmental stimuli including hydration, temperature, pH, and the presence of ions and proteins, or to external stimuli such as ultraviolet (UV) light or a magnetic field (Preiss and Bothun 2011; Paasonen et al. 2010; Malmsten

2007; Nguyen et al. 2011; Nguyen et al. 2010; Yaghmur et al. 2011a; Angelov et al. 2007; Fong et al. 2010). Therefore, there is increasing interest in basic and applied research to characterize such self-assembled nanoscale assemblies also under nonequilibrium conditions.

In general, most of the experimental studies for characterization of lipidic self-assembled soft nanoobjects in basic and applied research have been, and still are, performed under equilibrium conditions. These investigations include mapping binary lipid/water systems and characterizing the effect of solubilizing hydrophilic, hydrophobic, and amphiphilic guest molecules on various self-assembled nanostructures (Yaghmur and Glatter 2009; Kaasgaard and Drummond 2006; Larsson 1989; Yaghmur et al. 2005, 2010a; de Campo et al. 2004; Luzzati 1997). However, over the last 30 years, great efforts have been also invested in probing nanoscale structures under nonequilibrium conditions.

In the late 1980s, in parallel with the establishment of third-generation synchrotrons, first millisecond time-resolved (TR) X-ray measurements in different SAXS beamlines were put into practice (Laggner 1988; Caffrey 1989). Especially the study of rapid temperature-driven transitions in lyotropic liquid-crystal systems attracted great attention (Tate and Gruner 1989; Laggner and Kriechbaum 1991). Recent applications of infrared (IR) laser-induced temperature-jump (T-jump) and fast heat-conductive temperature-drop experiments on low-density lipoprotein dispersions are summarized by Prassl et al. (2008). Similar heat-conductive T-jump and T-drop experiments were also performed for studying the nonequilibrium liquid-crystalline structures of glycerol monooleate (MO) and phytantriol (PHYT) systems in excess water (Dong et al. 2010). Dynamic investigations on the structure and mechanics of biomimetic model membrane systems are still indispensable for understanding the geometric/topological relationship during phase transitions such as in membrane fusion processes (Yaghmur et al. 2008, 2010b; Dong et al. 2010; Angelov et al. 2009). Advanced developments in the field of pressure jump (p-jump) perturbations (Steinhart et al. 1999; Winter and Jeworrek 2009; Brooks et al. 2011), and especially the incorporation of microfluidic devices in SAXS beamline setups, have lately contributed to this research area by providing new experimental opportunities (Narayanan 2009; Toft et al. 2008). Chemical processes can be observed in situ by integration of a batch reactor connected to a flow-through capillary as applied for, e.g., investigations on real-time formation of mesoporous materials or time-resolved observations of digestion processes (Ågren et al. 1999; Teixeira et al. 2011; Warren et al. 2011; Salentinig et al. 2011). Furthermore, combination of SAXS with a stopped-flow apparatus or a remote-controlled syringe system allows new insights into structural transition mechanisms and kinetic pathways under nonequilibrium conditions (Yaghmur et al. 2008, 2011a, b; Rappolt et al. 2006; Alam et al. 2011; Salentinig et al. 2011).

Another interesting example of the need for characterizing the dynamic behavior of self-assembled systems is the formation of stimuli-responsive liposome assemblies (Preiss and Bothun 2011; Paasonen et al. 2007, 2010; Yaghmur et al. 2010b; Namiki et al. 2011; Pornpattananangkul et al. 2010). These present a new and unique family of delivery systems that are designed for initiating and controlling drug release on demand as a response to external stimuli such as a light source or a magnetic field. Investigations under nonequilibrium conditions are especially helpful in optimizing the utilization of these formulations. Understanding how to initiate and control the drug release underscores the need for real-time monitoring of the changes in the nanostructure, the permeability, and the stability upon exposure to an external stimulus (Paasonen et al. 2010; Yaghmur et al. 2010b). In addition to liposomes, there is growing interest also in introducing stimuli-responsive inverted-type nonlamellar liquid-crystalline phases (Fong et al. 2010; Yaghmur et al. 2012).

Recent research studies have been especially focused on mimicking the conditions of direct exposure of lipidic drug nanocarriers to a biological environment (Yaghmur et al. 2008, 2010b, 2011a, b; Paasonen et al. 2010; Warren et al. 2011; Tilley et al. 2011; Vandoolaeghe et al. 2009; Mulet et al. 2009; Alam et al. 2011). For instance, we used a combination of synchrotron SAXS with remote-controlled addition of buffer to monitor the dynamic behavior of local anesthetic-loaded preformulations in situ (Yaghmur et al. 2011a). In this work, the model drug bupivacaine (BUP) was solubilized in *low-viscous* water-containing preformulations based on an inverted-type micellar ($L_2$) solution or an inverted type of hexagonal ($H_2$) phase. We investigated also the in vitro release properties of BUP from the in situ-formed inverted-type liquid-crystalline phases and microemulsions upon hydration (Yaghmur et al. 2012). The obtained results emphasize the important role of pH and partial replacement of glycerol monooleate (GMO) by medium-chain triglycerides (MCT) in modulating both the self-assembled nanostructure and the in vitro drug release profiles.

In this review, key aspects of different studies performed under nonequilibrium conditions are discussed. Its scope is restricted to recent investigations performed by the combination of SAXS with other techniques for characterizing the formation of self-assembled systems in situ and investigating the dynamics of structural transitions in the millisecond to second time range.

## Opportunities in time-resolved synchrotron light-based investigations

Current views on the formation and structural response of self-assembled systems imply a great variety of dynamic aspects. These range from the dynamic phase behavior of lipids upon exposure to biological environmental stimuli and the structural variations of lipidic nanoparticles in the presence of a model pharmaceutical cargo, to material exchange between different lipidic systems and fusion processes. The structural depiction of these courses of action calls for synchrotron time-resolved (TR) diffraction methods (Angelova et al. 2012; Narayanan 2009). The vast amount of biophysical studies on the lyotropic phase behavior of lipids concerns temperature- and pressure-jump (T- and p-jump) studies.

The fastest T-jump facility presently available for TR-SAXS studies (Laggner et al. 2005) on aqueous dispersions is installed at the Austrian SAXS station (Amenitsch et al. 1998). This T-jump system features as a core element an erbium glass IR laser (Rapp and Goody 1991). The laser pulse (commonly 2 ms and in Q-switch mode 1 μs long) is deposited onto a capillary sample holder, facilitating T-jumps on the order of 10–20 °C (Fig. 1a), corresponding to heating rates of 10,000 °C/s. For the investigation of liquid-crystalline phase transitions, this means that transitions can be induced within tens of microseconds. Because changes in the water content, for instance in multilamellar vesicles (MLVs), typically last a few seconds (Pabst et al. 2000), it is hence possible to decouple the heat-induced lipid chain melting events from water diffusion processes. In this manner, it was possible to directly probe, e.g., the membrane rigidity of phosphatidylcholine bilayers as a function of cholesterol content (Rappolt et al. 2003). While the membrane fluidization capability is greatest at 5 mol% cholesterol, the maximum membrane thinning after the T-jump reduces drastically at higher cholesterol content (Fig. 1b).

Moreover, the transition pathways far from equilibrium can be investigated with T- and p-jump techniques (Laggner et al. 2005; Winter and Jeworrek 2009). This is of utmost importance for relevant life-science studies and pharmaceutical applications, since biological reactions and also drug administration processes usually take place under nonequilibrium conditions. Phase heterogeneity (Rappolt et al. 2000) and the formation of intermediate phases (Laggner and Kriechbaum 1991; Squires et al. 2005) are often obscured near equilibrium, but become accessible under extreme temperature or pressure rates. In Fig. 1c, an example for rapid TR X-ray diffraction plots during the cubic gyroid (G, *Ia3d* symmetry) to diamond (D, *Pn3m* symmetry) phase transition induced by a rapid pressure drop is displayed. Two intermediate phases are labeled with (a) and (b), in which the latter, longer-living intermediate, has been identified as $H_2$-phase. A similar experiment was conducted by rapid hydration of the initial cubic G-phase (Rappolt et al. 2006). Again, an intermediate, likely $H_2$-phase, was observed, and also here the geometrical/topological relationships were discussed in detail. On the left-hand side of Fig. 1d, electron density maps displaying minimal surfaces of the G- and D-phases are shown, while on the right-hand side the corresponding water channel networks are depicted. The maximum electron density maps are displayed in viewing direction perpendicular to the (220)-planes (G-phase) and perpendicular to the (111)-planes (D-phase), respectively, which are believed to form an epitaxial relationship (Rappolt et al. 2006).

Further important for the biophysical characterization of lipidic systems is the investigation of their dynamic mechanical behavior. Phase transitions can, for instance, also be induced by application of shear stress (Porcar et al. 2002; Seddon et al. 2011), and membrane pore formation couples to acoustic irradiation (Deng et al. 2004). In the subsequent sections, and on the background of recently published own works, we especially review nonequilibrium perturbation methods that can become extremely useful for drug delivery research studies. An overview of presented techniques applied in combination with synchrotron SAXS is given in Table 1.

## TR-SAXS investigations on light-activated vesicles

Stimulus-responsive nanocarriers have high potential for application in controlled drug targeting and release on demand (Preiss and Bothun 2011; Angelova et al. 2012; Yaghmur et al. 2010b; Paasonen et al. 2010; Fong et al. 2010, 2012). For instance, incorporation of temperature-sensitive gold nanoparticles (NPs) in liposomes makes heat-triggered drug release possible, and this at a specific administration site within a controllable time (Paasonen et al. 2010; Yaghmur et al. 2010b; Preiss and Bothun 2011). These NP-loaded vesicles respond to an external stimulus such as UV light or near-infrared laser pulses by the occurrence of light-induced "hot spots of NPs," which immediately induce heat transfer to the surrounding lipid/water material (Paasonen et al. 2010; Preiss and Bothun 2011; Yaghmur et al. 2010b; Wu et al. 2008). The actual drug release is then triggered by a heat-driven increase of the membrane permeability. This approach has established important liposomal compositional and structural variables that control the membrane permeability and the release of pharmaceutical cargo on demand (Paasonen et al. 2007, 2010; Yaghmur et al. 2010b; Fong et al. 2009).

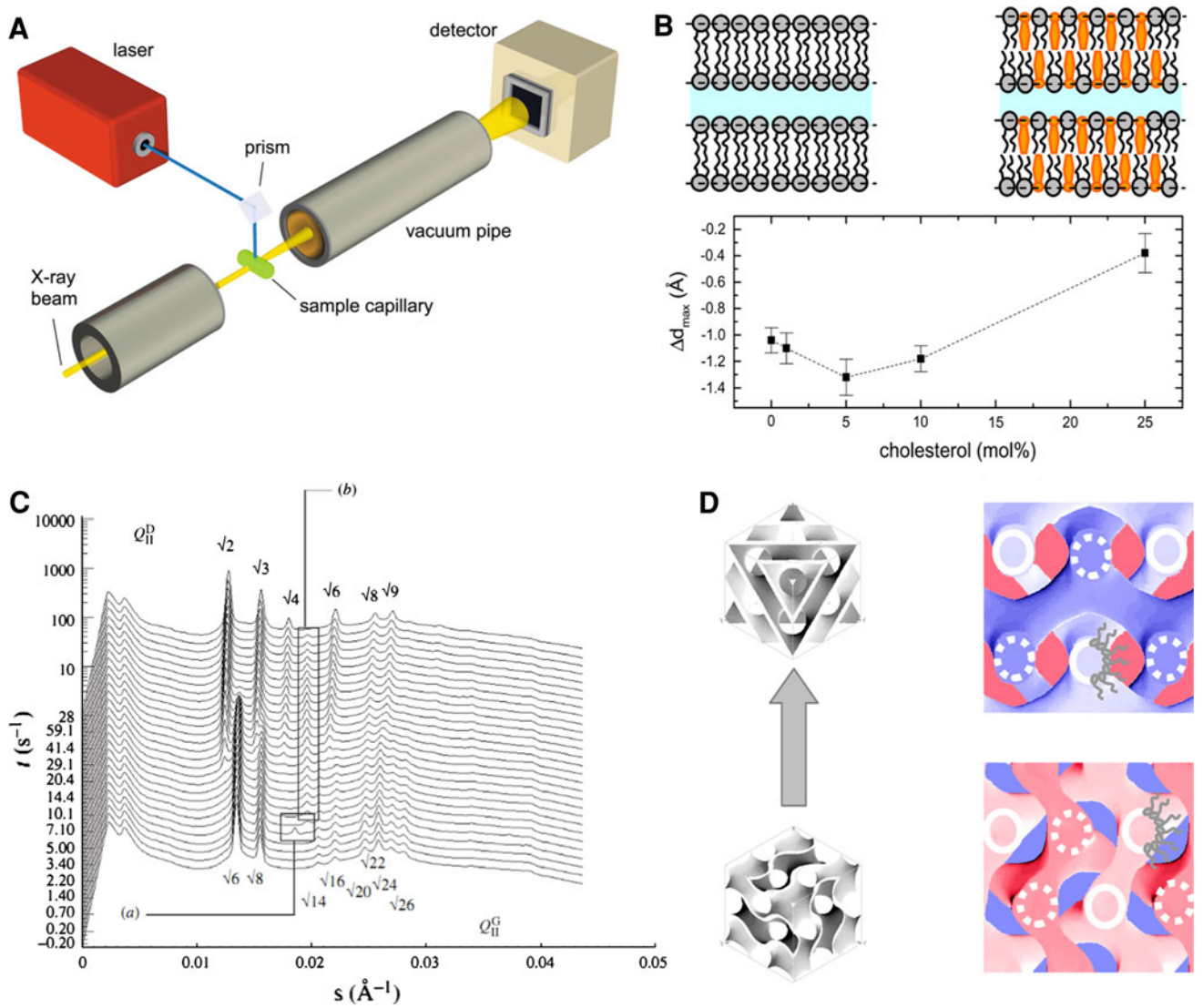


**Fig. 1** T- and p-jump experiments with synchrotron SAXS on lyotropic liquid-crystal systems. **a** Setup of a T-jump experiment using an erbium glass laser [figure reproduced with permission (Orthen et al. 2004)]. **b** Probing palmitoyl-oleoyl-phosphatidylcholine bilayer fluidity as a function of cholesterol content by applying 2 ms laser flash-induced T-jumps from 10 to 13 °C. Bilayer models basing on structural parameters deduced from X-ray diffraction experiments without and with cholesterol are depicted on *top*. The maximum membrane thinning $\Delta d$ is shown below [figure taken from Rappolt et al. (2003) with permission]. **c** TR-SAXS diffraction plots showing the cubic G- to D-phase transition induced at 59.5 °C by a rapid pressure drop of 600 to 240 bar [figure taken from Squires et al. (2005) with permission]. **d** The same phase transition induced by rapid water injection to the G-phase of monoolein. Electron density maps of the minimal surface (*left side*) and the water channel network (*right side*) are presented before and after hydration [figures adapted from Rappolt et al. (2006)]

In two recent studies, high permeability was achieved from liposomes having different lipid composition by induced membrane topology changes from planar → tubular monolayers (Yaghmur et al. 2010b), and geometry changes from rippled → planar lamellae (Paasonen et al. 2010). The release mechanism was triggered in both cases by UV light at $\lambda = 365$ nm, and simultaneous nanostructure investigations with synchrotron SAXS were carried out. In Fig. 2, the optothermally induced structural changes in multilamellar lipid vesicles (MLVs) of N-methylated dioleoylphosphatidylethanolamine (DOPE-Me) loaded with hydrophilic gold NPs with size of 4 nm are shown. The structure conversion pathway from vesicles with fluid lamellar ($L_\alpha$) membranes to $H_2$-phase through an intermediate state of uncorrelated fluid bilayers during in situ UV activation was observed (Yaghmur et al. 2010b). The experiments demonstrated that, depending on the UV lamp intensity (i.e., on the heat load), the $L_\alpha - H_2$ phase transition can be obtained in a few seconds, and further, 1 min after switching off the UV lamp, the nanostructure retransforms

**Table 1** Rapid perturbation techniques providing opportunities in biophysics (first 3 methods) and drug delivery research (last 4 methods)

| Perturbation method | Opportunities | References |
|---|---|---|
| Infrared laser or thermoelectric T-jump; heat-conductive T-jump (drop) by rapid injection into preheated (precooled) sample holders | Detection of intermediate phases<br>Geometric/topological studies<br>Decoupling of heating events from water diffusion | Laggner and Kriechbaum (1991), Laggner et al. (2005), Conn et al. (2006), Prassl et al. (2008), Dong et al. (2010) |
| Pressure jump | Detection of intermediate phases<br>Geometric/topological studies<br>Dynamic bidirectional phase-transition investigation | Steinhart et al. (1999), Winter and Jeworrek (2009), Brooks et al. (2011) |
| Shear stress; acoustic irradiation (ultrasound) | Dynamic mechanical properties of lipidic systems | Porcar et al. (2002), Seddon et al. (2011), Deng et al. (2004) |
| Stop-flow rapid mixing (fast processes) and vortex mixing (slow processes) | Mixing experiments with solutions and dispersions<br>Probing environmental changes (pH, salinity)<br>Interparticle mixing | Yaghmur et al. (2008), (2011b), Narayanan (2009), Panine et al. (2006), Weiss et al. (2005), Alam et al. (2011), Tilley et al. (2011) |
| Rapid injection/immersion | Hydration experiments on viscous liquid crystals or other viscous formulations<br>Probing environmental changes (hydration, pH, salinity) | Rappolt et al. (2006), Yaghmur et al. (2011a) |
| Flow-through capillary connected to a sample reaction cell | Following process operations<br>Product mixing<br>Chemical reactions<br>Self-assembly, crystallization | Ågren et al. (1999), Teixeira et al. (2011), Warren et al. (2011), Salentinig et al. (2011) |
| External physical stimuli such as flash lamps, UV light source, and magnetic field | Triggering of stimuli-responsive materials<br>Rapid release of caged compounds<br>Light-activated drug release<br>Magnetically targeted drug delivery systems | Rapp and Goody (1991), Yaghmur et al. (2010b), Paasonen et al. (2010), Namiki et al. (2011) |

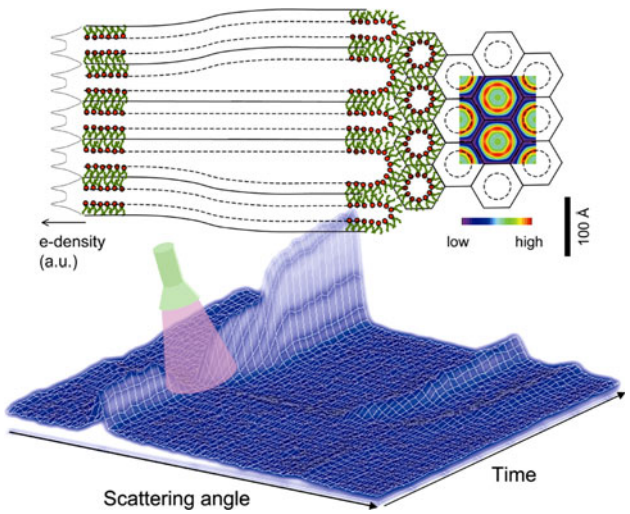


**Fig. 2** Optothermally induced structural changes in MLVs loaded with hydrophilic gold NPs. Synchrotron TR-SAXS experiments combined with UV light source irradiation demonstrated that the structure pathway from the fluid lamellar ($L_\alpha$) phase to an inverted hexagonal ($H_2$) phase passes through an intermediate state of uncorrelated membranes. The electron density profile of the $L_\alpha$-phase is shown at the *far left*, and the electron density map of the $H_2$-phase is shown on the *far right*. For the $H_2$-phase, the electron density values are *color coded* [figure adapted from Yaghmur et al. (2010b)]

into MLVs. Thus, in perspective, this stimulus-responsive material could in principle even serve as a drug deposit that provides repetitive on-demand drug release. A detailed description of the molecular rearrangements during the transition from the well-ordered MLVs in the fluid $L_\alpha$-phase to the $H_2$-phase via the formation of the intermediate phase of uncorrelated fluid bilayers was put forward in our recent study (Yaghmur et al. 2010b).

## In situ monitoring of nanostructures by coupling rapid mixing to TR-SAXS investigations

In recent investigations, a structural mechanism for how $Ca^{2+}$ ions at low concentrations induce the *recrystallization* of dioleoylphosphatidylglycerol (DOPG)/monoolein (MO) vesicles or a molten disordered sponge-like ($L_3$) phase to form well-ordered one-, two-, or three-dimensional nanostructures was proposed (Yaghmur et al. 2008, 2011b). A schematic description of these extremely fast nonequilibrium disordered–ordered structural transitions within the milliseconds to seconds range is illustrated in Fig. 3a. For a series of aqueous dispersions containing

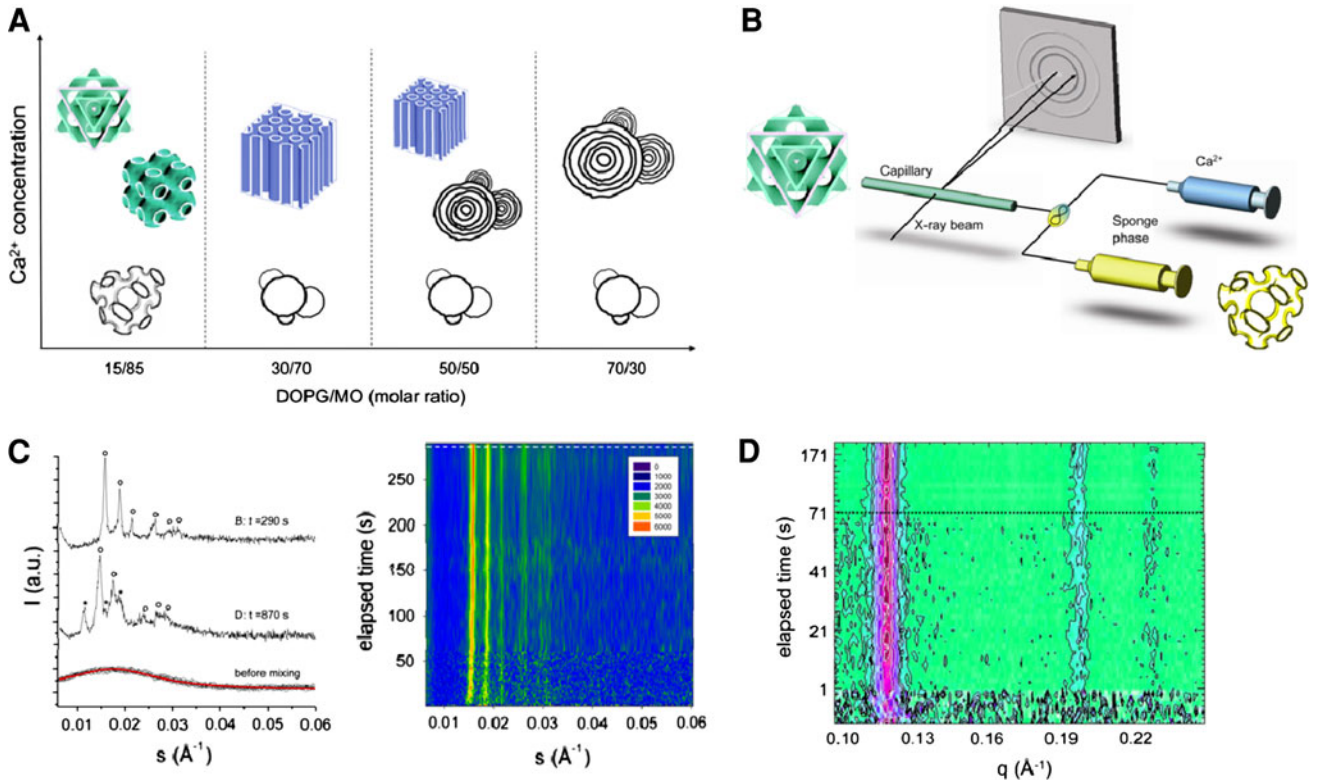


**Fig. 3** **a** Schematic illustration of the in situ calcium-triggered formation of well-ordered nanostructures: the two $V_2$-phases with symmetries *Pn3m* and *Im3m*, the $H_2$-phase, and the $L_\alpha$-phase. **b** Schematic illustration of the setup combining synchrotron SAXS with a stopped-flow apparatus. In the stopped-flow apparatus, one syringe contained a buffer with $Ca^{2+}$ ions, whereas the other was filled with DOPG/MO-based aqueous dispersion. **c** Selected SAXS patterns for three investigations at 50 °C: $L_3$-phase in the absence of $Ca^{2+}$ ions, and two examples of the in situ formation of bicontinuous cubic phases. On the *right*, the *contour plot* clearly displays the characteristic reflection patterns of the *Pn3m* phase. **d** The very fast calcium-triggered $H_2$-phase formation at 50 °C upon rapidly mixing DOPG/MO vesicles with low concentration of $Ca^{2+}$ ions is presented in a SAXS *contour plot* [figures adapted from Yaghmur et al. (2008, 2011b)]

different DOPG/MO ratios, in situ monitoring of these intriguing transitions was achieved by the combination of synchrotron SAXS with a stopped-flow apparatus (Fig. 3b) (Yaghmur et al. 2008, 2011b). The obtained clear experimental evidence on the strong and fast binding of $Ca^{2+}$ ions to the negatively charged DOPG/MO membrane shows the important role of the lipid composition, the investigated $Ca^{2+}$ ion concentration, and the investigated temperature in forming well-ordered multilamellar or nonlamellar phases. Moreover, different static SAXS measurements demonstrated the importance of structural analysis of self-assembled systems under *realistic* excess of water conditions (Yaghmur et al. 2008). The removal of excess water to form lipidic pellets prior to SAXS experiments can affect the nanoscaled structures.

In Fig. 3, two examples on the in situ monitoring of the direct calcium-triggered $L_\alpha$–$H_2$ (panel d) and $L_3$ to inverted-type cubic ($V_2$) phase (panel c) transitions are presented. In these studies, it is important to remark that the fast binding of $Ca^{2+}$ ions to the negatively charged DOPG/MO membrane induces rapid dehydration and concomitant condensation of the phospholipid bilayer membrane due to the screened electrostatic repulsive forces. It is intriguing to monitor in situ the very fast reordering of the lipids in these self-assembled structures and to observe the ease of tuning rapidly the membrane curvature as soon as the vesicles or $L_3$-phase are exposed to $Ca^{2+}$ ions (Yaghmur et al. 2008, 2011b). It should be noted that the first stages of binding of this divalent cation to the negatively charged DOPG molecules are undetectable in the investigated time window, as they take place in the nano- to millisecond range. This calls in the future for designing SAXS methods coupled to rapid mixing with nanosecond time resolution (see "Conclusions and perspectives").

The combination of SAXS experiments with rapid mixing is important for learning more on the interaction mechanism of metal ions with biologically relevant lipids under conditions mimicking the biological environment and for further improved understanding of the biological role of $Ca^{2+}$ ions in living cells.

### TR-SAXS for in situ characterization of stimulus-responsive drug-loaded formulations

A practical strategy for controlled release of drugs from potentially safe and biocompatible lipidic formulations at the administration site is the use of *low-viscous* stimulus-responsive drug preformulations, which are transformed to inverted-type highly organized hierarchical structures (such as nonlamellar $H_2$ and $V_2$ nanostructures) upon exposure to the biological environment (Yaghmur et al. 2011a; Malmsten 2007; Chang and Bodmeier 1998; Norling et al. 1992). Optimal utilization of such delivery methods requires full understanding of the dynamics of these preformulations by mimicking the conditions of their direct exposure to excess water under physiological conditions (pH 7.4, 37 °C) and also evaluating their drug release properties.

In our recent investigations (Yaghmur et al. 2011a), observation of the structural events upon exposure of *low-viscous* water-containing preformulations of inverted-type micellar solution ($L_2$) or an $H_2$-phase containing the local anesthetic bupivacaine (BUP) to excess buffer was achieved by the combination of TR-SAXS experiments with remote-controlled addition of buffer (Fig. 4a). Figure 4 shows also two examples on the performed TR-SAXS experiments as buffer with pH 7.4 was injected into

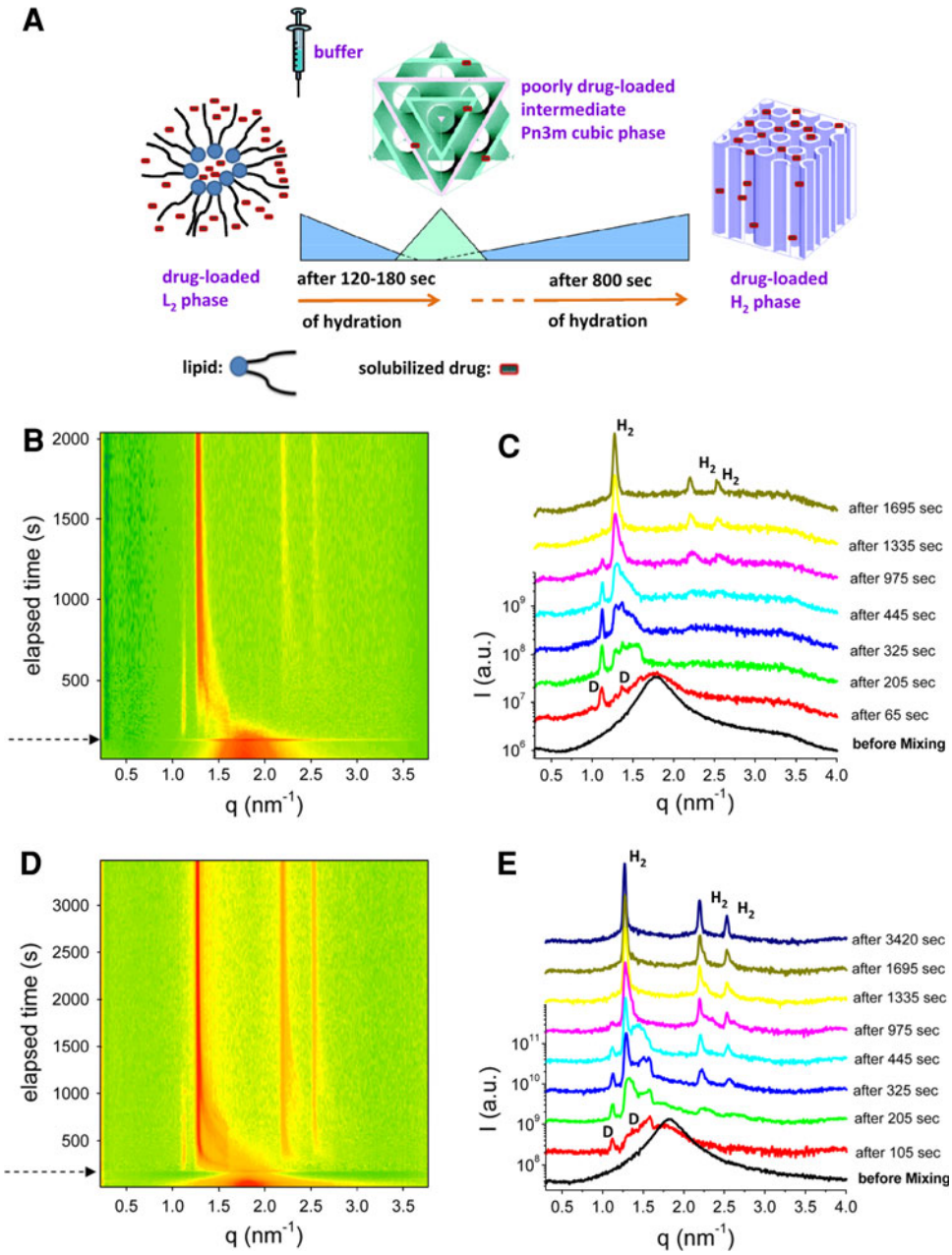


**Fig. 4** **a** In situ characterization of lipidic BUP-loaded formulations. In panels **b** and **d**, the contour plots display the fast hydration-triggered structural transitions (*dashed arrows* mark the arrival of the water front). SAXS patterns of all appearing phases at different elapsed times are shown in panels **c** and **e**. The two Bragg reflections of the intermediate *Pn3m* cubic phase are marked with "D" and those of the inverted hexagonal phase with "$H_2$" [figures adapted from Yaghmur et al. (2011a)]

two different BUP preformulations (low-water-containing $L_2$ solutions) that were prepared at pH 7.4 (panels b and c) or at pH 6.0 (panels d and e). The results obtained in both experiments show fast, interesting hydration-induced structural transitions: fast rearrangement of the lipid and the drug molecules in excess phosphate buffer followed by complete conversion of the preformulations to the fully hydrated $H_2$-phase (Fig. 4b–e). The equilibrium nanostructure is reached within about 1,000 s. The response of both preformulations was similar, involving a fast hydration stimulus that causes significant changes in the structure. The results suggest fast redistribution of BUP molecules between the polar interface and the hydrophobic regions, since the structural transition from the self-assembled preformulations to $H_2$-phase is not direct but involves the intermediate formation of a BUP-poor *Pn3m* cubic phase as an intermediate phase. Similar, Angelova and coworkers reported also on interesting studies on nonequilibrium and intermediate states in different lipid systems being attractive as nanocarriers (Angelov et al. 2007, 2009, 2011).

The in vitro rotating dialysis model was used to study BUP release after rapid exposure of GMO- or GMO/MCT-based preformulations to excess of phosphate buffer in the donor compartment of the in vitro rotating dialysis cell model at 37 °C (Yaghmur et al. 2012). It is evident from the evaluation of the release rates of BUP while varying the GMO/MCT weight ratio in these preformulations that the lipid composition plays a vital role in modulating the BUP release profiles in vitro. The release rate becomes faster with increasing MCT content. It is enhanced in the same direction as the observed *Pn3m* → $H_2$ → $L_2$ transition. The obtained data emphasize also the important role of pH on modulating both the self-assembled nanostructure and the in vitro drug release profiles. The reported pH sensitivity of the structure and the BUP release properties is most likely due to the change in affinity of the solubilized BUP to the lipid-based nanostructures as reflected in the determined lipidic partition coefficient between the GMO-based liquid-crystalline nanostructures and the phosphate buffer solution.

## Conclusions and perspectives

SAXS is one of the most powerful methods for probing the nanostructures of self-assembled systems. It is commonly used for structural characterization of various nanosystems that are promising for the formulation design of food and drug nanocarriers. In recent years, this growing interest has focused not only on their structural characterization at the nanoscale prior to use, but also on fully understanding their structural formation mechanism and dynamics as they are exposed either to an external physical stimulus or to different biologically relevant environments. There is promising progress in the development of SAXS coupling to other techniques (Table 1) for *real-time* monitoring of self-assembled systems in the millisecond to second range under nonequilibrium conditions. This helps also to improve understanding of the basic principles involved in their interaction with active biomolecules and metal ions. For instance, SAXS in combination with different techniques is auspicious in learning the characteristics and properties of stimuli-responsive assemblies attractive as nanocarriers for solubilizing and controlling the release of drugs, peptides, and other bioactive materials on demand.

Future perspectives concern mainly technical developments for delivering new and efficient experimental opportunities. The time resolution in p-jumps can be optimized by use of piezoelectric stack pistons to generate jumps up to 200 bar in 150 μs (see Brooks et al. 2011 and references therein). Similar Q-switched lasers can produce 1-μs pulses, but also here the energy release is reduced to a few hundred Joules, allowing T-jumps only on the order of 1 °C (Rapp and Goody 1991). Further, the T-jump technique using IR laser heating can be refined by the use of a second, fast triggering laser in continuous mode to keep the final temperature constant, i.e., to avoid passive cooling after the T-jump. It is worth pointing out that there are a number of emerging experimental setups that not only combine TR-SAXS with various sample manipulation techniques, but also include probing simultaneously other physical parameters. First, differential scanning calorimetry (DSC) X-ray cells allow medium to fast temperature ramps (1–30 °C/min), recording simultaneously the X-ray scattering patterns and the enthalpy changes of the specimen (Rappolt et al. 2008). Second, setups combining TR-SAXS with simultaneous spectroscopic measurements have been put into practice, e.g., the complementary technique Fourier-transform infrared spectroscopy (FTIR) was applied to the study of self-assembly of mesostructured films during dip-coating (Innocenzi et al. 2007). Last, fast biological and chemical reactions can be studied using continuous flow free-jet mixers (Marmiroli et al. 2009), pushing down the time limit to microsecond resolution, i.e., roughly three orders of magnitude faster than the common stop-flow apparatus. This relatively new mixing technique is attracting also the new community of upcoming X-ray free electron laser (X-FEL) source users (DePonte et al. 2011). It is clear that future X-FEL experiments designed for biological and pharmaceutical research will be a great challenge. This is a new tool with the ambitious plan of providing new insights into the fastest temporal behavior of even complex molecular systems: reactions with picosecond ($10^{-12}$ s) time resolution will become accessible.

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