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Bile Salt Micelles and Phospholipid Vesicles Present in Simulated and Human Intestinal Fluids: Structural Analysis by Flow Field–Flow Fractionation/Multiangle Laser Light Scattering

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ABSTRACT

Knowledge about colloidal assemblies present in human intestinal fluids (HIFs), such as bile salt micelles and phospholipid vesicles, is regarded of importance for a better understanding of the *in vivo* dissolution and absorption behavior of poorly soluble drugs (Biopharmaceutics Classification System class II/IV drugs) because of their drug-solubilizing ability. The characterization of these potential drug-solubilizing compartments is a prerequisite for further studies of the mechanistic interplays between drug molecules and colloidal structures within HIFs. The aim of the present study was to apply asymmetrical flow field–flow fractionation (AF4) in combination with multiangle laser light scattering in an attempt to reveal coexistence of colloidal particles in both artificial and aspirated HIFs and to determine their sizes. Asymmetrical flow field–flow fractionation/multiangle laser light scattering analysis of the colloidal phase of intestinal fluids allowed for a detailed insight into the whole spectrum of submicron- to micrometer-sized particles. With respect to the simulated intestinal fluids mimicking fasted and fed state (FaSSIF-V1 and FeSSIF-V1, respectively), FaSSIF contained one distinct size fraction of colloidal assemblies, whereas FeSSIF contained 2 fractions of colloidal species with significantly different sizes. These size fractions likely represent (1) mixed taurocholate-phospholipid-micelles, as indicated by a size range up to 70 nm (in diameter) and a strong UV absorption and (2) small phospholipid vesicles of 90-210 nm diameter. In contrast, within the colloidal phase of the fasted state aspirate of a human volunteer, 4 different size fractions were separated from each other in a consistent and reproducible manner. The 2 fractions containing large particles showed mean sizes of approximately 50 and 200 nm, respectively (intensity-weighted mean diameter, D_z), likely representing mixed cholate/phospholipid micelles and phospholipid vesicles, respectively. The sizes of the smaller 2 fractions being below the size range of multiangle laser light scattering analysis (<20 nm) and their strong UV absorption indicates that they represent either pure cholate micelles or small mixed micelles. Within the colloidal fraction of the fed-state human aspirate, similar colloidal assemblies were detected as in the fasted state human aspirates. The observed differences between SIF and HIF indicate that the simulated intestinal fluids (FaSSIF-V1 and FeSSIF-V1) represent rather simplified models of the real human intestinal environment in terms of coexisting colloidal particles. It is hypothesized that the different supramolecular assemblies detected differ in their lipid composition, which may affect their affinity toward drug compounds and thus the drug-solubilizing capabilities.

Introduction

Today's drug discovery screening programs show a tendency toward more lipophilic drug candidates because of the fact that high receptor affinity is partly governed by hydrophobic interactions. This leads to a large fraction of drug candidates being classified as class II in the Biopharmaceutics Classification System (BCS), which all exhibit poor solubility and high permeability.[1,2] For this class of compounds, oral absorption is thus primarily hampered by their limited solubility and dissolution rate. In the human gastrointestinal tract, secretion of bile salts is an important physiological mechanism rendering fatty food components more

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soluble via micellar solubilization. Via the same mechanism, the dissolution rate and apparent solubility of lipophilic drugs are increased, which affects their absorption. This is also regarded one of the reasons underlying "food effects" when ingesting a meal concomitant with a lipophilic drug.[3] The exact mechanisms that determine the fate of a given drug from its release from a pharmaceutical formulation all the way into the blood or lymph are not fully understood though.

It is commonly agreed, however, that simulated intestinal fluids (SIFs), which contain bile salt(s) and phospholipid(s), possibly in combination with other lipids, allow for a more biomimetic *in vitro* dissolution testing of drug formulations than mere aqueous systems. In recent years, considerable efforts have been undertaken to reveal individual molecular constituents present in human intestinal fluids (HIFs) at different nutritional states and to adopt "biorelevant" compositions in SIFs.[4-9] In essence, artificial media are judged as more or less "biorelevant" based on their performance as "solvents" for lipophilic drugs.[10] Such comprehension of biomimetic media as "uniform solvents," however, has been hypothesized to be too simplistic.[11] Theoretically, there may arise different colloidal self-associates from the constituents typically present in intestinal fluids such as cholate micelles, mixed cholate/phospholipid micelles, and phospholipid vesicles. Model studies have demonstrated that mixed micelles transit into phospholipid vesicles on dilution.[12] Vogtherr et al.[13] reported the coexistence of both, mixed lecithin−taurocholate micelles and pure taurocholate micelles in FaSSIF, which, interestingly, displayed differences in solubilizing capacity/affinity toward the specific amphiphilic drug investigated. The aim of the present study thus was to establish an approach to separate different colloidal species present in intestinal fluids from each other according to their sizes in an analytical to small preparative manner.

Previous attempts to characterize the colloidal structures in artificial intestinal fluids (SIF) have been carried out using a combination of cryogenic transmission electron microscopy (Cryo-TEM) and photon correlation spectroscopy (PCS)[11,14] and recently with diffusion ordered spectroscopy (DOSY) NMR.[13] Kloefer et al. applied Cryo-TEM imaging and reported rather monodisperse small colloids in FeSSIF, which were hypothesized to represent small mixed taurocholate/phospholipid micelles. For FaSSIF, they showed the copresence of 2 types of structures, likely mixed micelles and small vesicles/liposomes.[14] Furthermore, the phase behavior and aggregation states of dietary lipids during duodenal digestion have been studied excessively, both *in vitro* and *ex vivo* (for a recent review, see Phan et al.[15]).

To the best of our knowledge, only few studies have made an attempt to explore colloidal species within aspirated HIF[16,17] by Cryo-TEM.

Considering the inherent limitations of imaging techniques, this field of research would benefit from an alternative approach to fully characterize HIFs regarding colloidal species with respect to their sizes and eventually morphology. Such an attempt is undertaken in this study by applying combined AF4/multiangle laser light scattering (MALLS) to colloidal dispersions of both SIF and HIF, where fractionation of any submicron species present, based on their hydrodynamic sizes, is followed by online analysis of their sizes and shape factors using multiangle laser light scattering. Furthermore, the method should allow for collection of the respective particle fractions for subsequent offline analysis and further characterization.

Because separation of particles occurs by applying a crossflow to the channel flow, the technique is a gentle, nondestructive way of separating, for example, micelles and liposomes in a mixed dispersion. After initial, vertical separation (i.e., focus step) the particles are eluted, which results in a horizontal separation as well, because of the parabolic flow profile of the eluent. This way, smaller particles elute before comparable larger ones and the resulting fractogram, produced by MALLS, display this as intensity peaks of light scattered from the individual size fractions of the particles injected.

The AF4/MALLS approach has previously shown excellent capability to fractionate colloidal particles into distinct size fractions and determine their size distribution[18-23] for disperse liposomal samples. Preliminary experience with fractionation of micellar species was gained during fractionation of colloidal species arising from amorphous solid dispersions on their dispersion in aqueous media.[24,25]

Materials and Methods

Materials

HIF samples, of both fasted and fed nutritional state, were kindly provided by Prof. Patrick Augustijns and his group (Leuven, Belgium). The fluids were collected from the duodenum of healthy volunteers during a clinical study at the University Hospitals Leuven,[9] approved by the Committee of Medical Ethics (ML7918). Fluids were sampled during 90 min after intake of 240 mL of water. To simulate the fed state, 400 mL of Ensure Plus was ingested 20 min before the intake of water. After aspiration, samples were pooled per volunteer and nutritional state and stored at −26°C. Sodium azide (≥99.5%), sodium chloride (≥99%), sodium taurocholate hydrate (≥97%), and sodium dihydrogen phosphate dihydrate (≥98%) were purchased from Sigma-Aldrich Denmark ApS (Brøndby, Denmark). Sodium hydroxide (≥98%) was purchased from Merck A/S (Hellerup, Denmark). Purified water was obtained with a laboratory water purification system (Milli-Q® Advantage A10® system; Merck-Millipore, Merck A/S, Hellerup, Denmark). The FaSSIF/FeSSIF original powders were purchased from *Biorelevant.com*.

Preparation of SIF Media

The desired volumes of FaSSIF-V1 or FeSSIF-V1 were prepared in volumetric flasks (A grade) following the manufacturer's instructions. After mixing the respective powder with its relevant buffer solution, the resulting dispersion was allowed an equilibration time of at least 2 hours at room temperature, with occasional gentle shaking, until further use.

Preparation and Characterization of HIF Media Constituents

The collection of fasted and fed state HIF (FaHIF and FeHIF) at KU Leuven was described in Materials. For the purpose of this study, frozen samples of intestinal fluids from one individual only (designated as human volunteer no. 20 in the preceding article by Riethorst et al.[9]) were shipped from KU Leuven to SDU Odense on dry ice.

On arrival, the FaHIF and FeHIF samples were thawed and divided into aliquots, refrozen and stored in a freezer (−20°C) until further usage. After thawing of one or more aliquots at room temperature, the sample(s) were bench-top centrifuged (Eppendorf Centrifuge 5804 R, Hamburg, Germany) at 21°C for 90 min at 20.913 g, using a fixed-angle rotor. Subsequently, the supernatant (micellar phase) was collected for further analysis.

Photon Correlation Spectroscopy

The particle sizes of the supramolecular assemblies present in selected samples and collected fractions were measured using PCS (DelsaMax; Beckman Coulter Denmark ApS c/o, Copenhagen, Denmark) in backscattering mode at 25°C in a fixed position using