Evaluation of a method of preparation of lipid emulsions as a model for chylomicron-like particles

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Abstract

Chylomicron remnants can penetrate into the artery wall, where they can initiate atherogenesis. Since it is difficult to isolate these particles from human blood because of contamination with other lipoproteins, the use of lipid emulsions as chylomicron remnant-like particles (CRLP) has been proposed to study their metabolism. The present work was aimed to evaluate the methodology for the preparation of CRLP. Artificial chylomicrons were prepared by sonication of a lipid mixture and separated by density gradient centrifugation. Lipid classes were analyzed by HPLC and fatty acids by GC. Particle size was measured by dynamic light-scattering and the presence of apolipoprotein E by immunoblotting. The highest lipid content was found in the $60 < S_f < 400$ fraction, followed by the $S_f > 400$. This latter fraction presented the highest triacylglycerol concentration, which was dramatically reduced in the $20 < S_f < 60$ fraction. Fatty acid composition in triacylglycerol and phospholipids resembled that of the standards used with little modifications. The repeatability of the method was excellent, showing relative standard errors below 10%. The mean size of the $60 < S_f < 400$ and $S_f > 400$ fractions, was 195.1 nm and 347.8 nm, respectively. The lipid analysis showed that $S_f > 400$ particles resembled the composition of natural chylomicrons and the $60 < S_f < 400$ particles that of chylomicron remnants, being the range of particle size more homogeneous in the $60 < S_f < 400$ fraction. The present is a reliable method for the preparation of CRLP, but also native chylomicron-like particles, in terms of lipid composition and particle size.

Introduction

Dietary lipid components are absorbed by enterocytes and reassembled in chylomicrons, which are large triacylglycerol-rich lipoproteins (TRL) that are secreted into lymph and pass into the blood via the thoracic duct. Chylomicron particles then undergo rapid lipolysis by lipoprotein lipase in extrahepatic capillary beds, a process that removes some of the triacylglycerol (TAG) and leaves smaller chylomicron remnants, which deliver the remaining dietary lipids to the liver (Redgrave, 1983; Yu and Cooper, 2001). Chylomicron remnants contain apolipoprotein (apo) B48 as structural protein, secreted as an integral part of the parent chylomicrons, and apo E, which is acquired in the circulation and has a key role in their hepatic uptake (Yu and Cooper, 2001; Rohlmann, et al., 1998). Chylomicron remnants are strongly atherogenic as have been shown to penetrate the artery wall as efficiently as low-density lipoproteins (LDL), and to be retained within the sub-endothelial space where they can initiate the atherogenic process (Mamo and Wheeler, 1994; Proctor and Mamo, 1998; Grieve, et al., 1998; Yu and Cooper, 2001). Thus, in addition to their effects on plasma LDL concentrations, dietary lipids are able to influence atherogenic events in the artery wall directly during their transport from the intestine to the liver in chylomicron remnants (Mamo and Wheeler, 1994; Proctor, et al., 2002; Yu and Mamo, 2000). This is of clinical relevance, as there is an association between delay in chylomicron remnant clearance and the development of atherosclerosis (Karpe, 1999).

Since it is difficult to isolate chylomicron remnants from human blood because of contamination by other lipoproteins, a number of authors use chylomicron remnant-like particles (CRLP) as artificial model to study their metabolism. These particles mimic

the effects of physiological chylomicron remnants in macrophages and in several cell models (Batt, et al., 2004; Moore, et al., 2004) and provide better reproducibility of chylomicron remnants particle composition in comparison with postprandial particles isolated from human blood. Furthermore, lipophilic molecules, such as dietary lipid minor components, can easily be incorporated into the model particles, making it a suitable and convenient model to study lipid metabolism.

Redgrave and Maranaho (1985) firstly proposed a method for protein-free TAG-rich emulsions similar to commercial emulsions like Intralipid. They included a cholesterol ester to the Intralipid formula, which only contained TAG, cholesterol and phosphatidylcholine, as they believed that its absence could affect the particle metabolism. Since the first approximations, the method has been greatly improved and today, pH-adjusted buffers and other phospholipid classes are included in the particle at different concentrations (Lenzo, et al., 1988). Whereas the applicability of the model in experimental animals and cell cultures has been sufficiently assessed, the method for preparing CRLP has not been adequately evaluated. The final composition of the resulting particles has been analyzed by different indirect methods for each lipid class, like enzymatic kits for TAG and cholesterol, the o-phthalaldehyde procedure for cholesterol and phosphorus determination for phospholipids (Redgrave and Maranaho, 1985; Lenzo, et al., 1988; Diard, et al., 1994). However, the lipid composition of CRLP has not been systematically analyzed by means of a single method for the determination of all lipid classes contained.

It has been generally believed that sequential ultracentrifugation generates emulsions with homogeneous particle size (De Pascale, et al., 2006). Commonly, only the fraction

similar to chylomicron remnants is analyzed and it is presently not known if the lipid classes and fatty acids, as well as particle sizes, are equally distributed among all fractions or if the preparation process, including the size fractionation, affects their distribution. Variations in the lipid composition of the particle, like fatty acids of surface lipids, may have pronounced effects on their metabolism (De Pascale, et al., 2006). However, the fatty acid composition of CRLP has been rarely analyzed (Hultin, et al., 1995) and never validated. Newly available techniques, like HPLC to an evaporative light-scattering detector or dynamic light scattering (DLS) allow a systematic validation of the lipid composition of CRLP and size.

The aim of the present work was to evaluate the methodology for preparation of artificial chylomicrons as a model of CRLP, by means of analyzing the lipid classes composition, their structural organization and the size of the particles resulting from the different fraction obtained in the preparation process. The method resulted to be particularly reliable for the $60 < S_f < 400$ fraction of CRLP, which is usually used as model of chylomicron remnants. Thus, for the first time, the methodology for CRLP preparation was adequately validated, and we consider that it can be employed with a high level of confidence.

Methods

Preparation of artificial chylomicrons

All lipid standards for preparation of artificial chylomicrons were supplied by Larodan Inc (Malmö, Sweden). Artificial chylomicrons were prepared using a lipid mixture containing 70% triolein, 2% cholesterol, 5% cholesteryl ester, and 25% phospholipids (Sigma-Aldrich, St. Louis, MO, USA) in 0.9% NaCl in tricine buffer (20 mmol/L, pH 7.4) as described by Redgrave and Maranhao (1985). The composition of the initial phospholipid moieties was: phosphatidylcholine 70.50%, lysophosphatidylcholine 6.88%, phosphatidylethanolamine 11.00%, phosphatidylinositol 2.58%, phosphatidylserine 2.58% and sphingomyelin 6.54% (Sigma-Aldrich, St. Louis, MO, USA). Lipids were sonicated by immersing a clean ultrasonic probe (Bandelin Electronics, Berlin, Germany) directly into the cell-solvent mixture and supplying the probe with 50 W for 20 minutes (5 minute bursts with 5 minute rest periods to allow for cooling) at 56°C. The resulting emulsion was brought to a density of 1.21 g/mL with KBr, layered under a stepwise density gradient (2.5ml d 1.065 g/ml, 2.5ml d 1.020 g/ml, 3ml d 1.006 g/ml) as described by Diard et al. (1994), and centrifuged at 17 000g for 20 minutes at 20°C (Beckman Optima L-90K centrifuge; Beckman Coulter, Palo Alto, CA, USA) in a SW41Ti swing-out rotor. The upper layer, which was an uneven emulsion of lipids (**Svedberg** flotation rate (Sf)>400) was then collected and stored under N₂ at -20 °C. The removed layer was replaced with an equal volume of NaCl solution (d=1.006 g/mL), and tubes were centrifuged at 70 000g for 1h (20°C) in a SW41Ti swing out rotor. The upper layer (60<Sf<400) was again then collected and stored under N₂ at -20 °C. The removed layer was replaced with an equal volume of NaCl solution (d=1.006 g/mL), and tubes were centrifuged at 274 000g for 16 hours (20°C) in a SW41Ti swing

out rotor. The upper layer ($20 < Sf < 60$) was again then collected and stored under N_2 at $-20^\circ C$. The remaining emulsion was also collected and stored under N_2 at $-20^\circ C$. This process was repeated six times for intra-assay assessment and in six different days for inter-assay assessment.

Lipid composition of artificial chylomicrons by HPLC-ELSD

Total lipids were extracted from 1 mL of $Sf > 400$, $60 < Sf < 400$, and $20 < Sf < 60$ particles following a modification of the method of Folch et al. (1957), using 2,6-di-tert-butyl-p-cresol (BHT) as antioxidant. The lipid content of the $Sf < 20$ fraction was too low to be accurately analyzed. The extracted lipids were redissolved in 1 mL of chloroform/methanol (2:1, by vol.) and preserved at $-20^\circ C$ until used. The lipid classes composition was determined as described by Perona and Ruiz-Gutierrez (2004). In brief, a 2695 Alliance liquid chromatograph (Waters, Milford, MA, USA) was used and was computer controlled by the Empower Pro System (Waters, Milford, MA, USA). Typically, 10 μL of lipids, dissolved in chloroform/methanol (2:1, v/v), were injected by an automatic sampler. Detection was accomplished with a light-scattering detector (ELSD 2420, Waters, Milford, MA, USA). The detector settings were kept constant in all experiments using an evaporation temperature of $40^\circ C$ and a nitrogen pressure of 60 psi. The separation of lipid classes was carried out on a Lichrosphere diol column (250 x 4.6 mm, 5 mm particle size (Merck, Darmstadt, Germany)). The column temperature was maintained constant at $30^\circ C$ throughout the experiments. The flow rate was 0.8 mL/min. Stock solutions containing 1 mg/mL of cholesterol oleate, triolein, cholesterol, monoolein and phosphatidylcholine (Sigma-Aldrich, St. Louis, MO, USA) were prepared in chloroform/methanol (2:1, v/v) and used for identification and quantification. Quintuple runs of six concentrations of these standards (0.25 to 1

mg/mL) were injected. Equations resulting from third-order regression curves of these standards, with correlation coefficients >0.999 were employed for quantification.

Triglyceride and Phospholipid separation and fatty acid composition

TAG and phospholipids were isolated by solid-phase extraction diol columns (Supelclean LC-Diol; Supelco, Bellefonte, PA, USA) using hexane:methylene chloride (9:1, v/v) as eluent. The first eluate containing TAG was evaporated to dryness under a stream of nitrogen and redissolved in hexane. The second fraction, containing phospholipids, was eluted with methanol, evaporated to dryness under a stream of nitrogen and redissolved in chloroform/methanol (1:1, v/v).

For fatty acid analysis, lipids were transmethylated using sodium methoxide in methanol (0.5%) and the resulting fatty acid methyl esters (FAME) analyzed by GC, using a model 5890 series II gas chromatograph (Hewlett-Packard Co, Avondale, USA) equipped with a flame ionization detector and a capillary silica column Supelcowax 10 (Supelco Co, Bellefonte, USA) of 60 m length and 0.25 mm internal diameter as previously described (Ruiz-Gutierrez, et al., 1992). FAME were identified by comparison of their retention times against those of standards and quantified by external standard using peak area integration.

Artificial chylomicron particle size

The size of $S_f > 400$ and $60 < S_f < 400$ particles was determined by DLS. This technique measures the hydrodynamic diameter and refers to particle diffusion within a fluid. The diameter obtained by this technique is that of a sphere that has the same translational diffusion coefficient as the particle being measured. A correlator is used to analyze the

variable intensity of light scattered from a sample in the time domain. A Zetasizer Nano ZS instrument (Malvern Instruments, Malvern, UK) was used (O'Neal, et al., 1998). The instrument uses a 10 mW helium-neon laser at 632.8 nm to excite the samples. Scattered light is collected at 173° using a non-invasive back scattering technology. The software of the instrument derives particle size from the correlator function. Results are expressed as the Z-average mean which is the harmonic intensity averaged particle diameter. For the study, all measurements were performed at 25°C, in triplicate.

Apolipoprotein transfer to artificial chylomicrons

The apolipoprotein transfer was achieved by incubation of the harvested particles ($60 < S_f < 400$) with the density greater than 1.020–g/mL fraction of human serum (1:2, vol/vol), prepared by ultracentrifugation and dialyzed before use (37°C with shaking for 4 h) (Hultin, et al., 1995). After incubation, the particles were ultracentrifuged (154 000g, 16h) under a NaCl solution ($d=1.006$ g/mL). The upper layer was collected and recentrifuged (254 000g, 5h) under a NaCl solution ($d=1.006$ g/mL) to isolate the CRLP fraction. The presence of apo E was determined by SDS-PAGE with Commassie blue stain and Western-blot. After delipidation CRLP ($60 < S_f < 400$) and human serum samples were run by SDS-PAGE. The gel was then electrophoretically transferred in an electroblotting unit to a polyvinylidene difluoride (PVDF) membrane. Anti-apo E antibody diluted in TBS-T containing 5% non-fat milk was used to detect the indicated protein. The appropriate horseradish peroxidase (HRP) and a conjugated secondary antibody were used and the positive antibody reaction was detected with a ChemiGenius Bio Imaging System. (Syngene, Cambridge, UK).

Statistical analysis

Results are expressed as means and standard error (SEM) (n=6). The significance of the differences among particle fractions was assessed by one-way ANOVA with unpaired two-tailed t-test for comparison of the means. Analyses were done with the Prism 4.0 (Graph-PAD Software, San Diego, USA) statistical package. Differences of $P < 0.05$ were considered statistically significant.

Results

Lipid composition of artificial chylomicrons

The total lipid content of artificial chylomicrons, calculated as the sum of all lipids measured, is depicted in figure 1. The highest lipid content was found in the 60<Sf<400 fraction, followed by the Sf>400 and the 20<Sf<60 fractions. Intra-assay repeatability (Figure 1a) was excellent, showing relative standard error (RSE) values lower than 2.5% for all fractions. Likewise, inter-assay repeatability was also good for the Sf<20 and Sf>400 fractions, with RSE values of 2.5% and 4.9%, respectively. However, a low inter-assay repeatability was found for the intermediate chylomicron fractions, being the RSE value for the 20<Sf<60 fraction 16.1% and that of the 60>Sf>400 fraction 13.3%.

Table 1 shows the lipid classes composition of artificial chylomicrons. Striking differences were found for the main lipid classes studied, TAG and phospholipids, among the three main fractions. Whereas the Sf>400 fraction presented a very high concentration of TAG (about 84 mg/100mg), this lipid class was dramatically reduced in the 20<Sf<60 fraction (between 13.4 mg/100mg to 36.4 mg/100mg). In contrast, the phospholipid content was significantly higher in the 20<Sf<60 fraction compared to the Sf>400 one. It was interesting to find monoacylglycerols (MAG) among the lipid classes determined despite not being added to the starting lipid mixture. The concentration was, nevertheless, low, ranging from 0.5 mg/100 mg to 2.0 mg/100mg. Intra-assay repeatability was excellent in the 60<Sf<400 and Sf>400 fractions, with RSE values between 2.0% and 8.2%, except for MAG and phospholipids in the Sf>400 fraction. The fraction with the highest density (20<Sf<60) showed a good repeatability for TAG and phospholipids (6.0% and 2.5% respectively) but not for cholesterol esters,

cholesterol and MAG (above 23%). Similar results were found for inter-assay repeatability, showing lower RSE values for TAG and phospholipids.

Fatty acid composition of artificial chylomicrons

Table 2 shows the fatty acid composition of the TAG contained in artificial chylomicrons. As expected, oleic acid (18:1, n-9) presented the highest concentration (about 90%) in all fractions studied, as triolein was the only TAG added to the starting mixture. However, relevant concentrations of myristic (14:0), palmitic (16:0) and stearic (18:0) were also found. Intra and inter-assay repeatability was excellent for oleic acid in all fractions (RSE<2.5%) and was poor for the other fatty acids found (ranging from 8.1% to 41.3%).

The fatty acid composition of artificial chylomicron phospholipids is presented in table 3. The content of stearic acid was significantly higher, whereas that of linoleic (18:2, n-6) and arachidonic (20:4, n-6) acids were lower, in the Sf>400 fraction. No significant difference was observed for the concentration of palmitic and oleic acids among fractions. Intra-assay repeatability was good for palmitic, stearic, linoleic and arachidonic acids (about RSE=10% or lower) but not for oleic acid (up to RSE=21.1% for the Sf>400 fraction). The inter-assay repeatability was good or excellent for all phospholipid fatty acids.

Particle size of artificial chylomicrons

Particle size was studied in the most lipid-concentrated fractions (60<Sf<400 and Sf>400) by DLS and the results are shown in Figure 2. The mean size was higher ($P<<0.001$) in Sf>400 particles compared to 60<Sf<400 particles (Figure 2a). In Sf>400 particle sizes ranged from 150-1 000 nm, whereas in the 60<Sf<400 fraction, particles

ranged from 100-500 nm (Figure 2b). The effect of a wider range of particle size in the fraction with the larger particles, was reflected in a higher area under the curve when the percentage intensity was plotted against the size (Figure 2c). These data show that the 60<Sf<400 fraction had a more homogeneous range of particle size.

Apolipoprotein transfer to artificial chylomicrons

Incubation of the harvested emulsions of the 60<Sf<400 fraction with human serum, led to the incorporation of a number of proteins to the artificial particles, as observed by SDS-PAGE (Figure 3). According to their molecular weight, the main band corresponded to albumin (66 kDa). The rest of the bands corresponded to proteins with molecular weights in the order of those expected for apolipoproteins, including apo E (34 kDa). Western-blotting of revealed specifically the presence of apo E in both CRLP and human serum (Figure 4).

Discussion

Chylomicrons and their remnants can penetrate in the arterial wall and trigger the development of atherosclerosis. However, as these TRL are difficult to isolate from human blood because of contamination by other lipoproteins, CRLP were proposed as artificial models to study their metabolism (Botham, et al., 2005). Although preparation of CRLP is simple and convenient, to date the method has not been appropriately validated. Using ultracentrifugation techniques, chylomicrons are commonly isolated from the $Sf > 400$ fraction, whereas chylomicron remnants and very-low-density lipoproteins (VLDL) have been separated in the $20 < Sf < 400$ fraction (Botham, et al., 2005). However, most authors use the $60 < Sf < 400$ fraction for CRLP, as it supposedly resembles the size and composition of chylomicron remnants, which are considered the most atherogenic of all TRL (Diard, et al., 1994; Moore, et al., 2004).

In the present study we analyzed the lipid composition of four density artificial chylomicron fractions, according to the **Svedberg** flotation rate: $Sf > 400$, $60 < Sf < 400$, $20 < Sf < 60$ and $Sf < 20$. The highest lipid content of all fractions studied was found in the $60 < Sf < 400$ fraction (Figure 1). The preparation method by particle fractionation through ultracentrifugation allowed that about half of the lipids used for the preparations **were** recovered in that fraction. Intra-assay repeatability was good for this fraction but the inter-assay RSE was relatively high, probably due to difficulties in collecting the fraction by aspiration from the ultracentrifuge tube. Only about 20% of the lipids were recovered in the $Sf > 400$ fraction, despite being the one with the largest particles, but showing an excellent repeatability. In the $Sf > 400$ fraction, the absolute inter-assay values were much higher than those observed for the intra-assay study, **that**

can compromise the reliability of the methodology if this fraction is being used. It is interesting to note, that studies in the postprandial state have shown that the TAG concentrations in TRL of that flotation rate can be two or three-fold higher than compared to the $Sf > 400$ and the $20 < Sf < 60$ fractions (Palmer, et al., 2005).

The $Sf > 400$ fraction presented the highest TAG concentration relative to other lipid classes, which was expected due to its lower density (Table 2). The TAG content in the $Sf > 400$ fraction was very similar to native chylomicrons that have not been yet hydrolyzed by lipoprotein lipase in the bloodstream (Karpe, 1999) and both the intra-assay and inter-assay repeatabilities were excellent ($RSE < 2\%$). The similarity of the $Sf > 400$ fraction of artificial chylomicrons to natural native chylomicrons was also true for the rest lipid classes, with a 2-3% of free cholesterol and 11-12% of phospholipids, although RSE values were higher for these lipid classes. Likewise, the $60 < Sf < 400$ fraction resembled the composition of chylomicrons remnants and VLDL, and the $20 < Sf < 60$ that of VLDL remnants or intermediate-density lipoproteins (IDL), with regard to the lipid classes composition (Cabello-Moruno, et al., 2007). In both fractions, TAG and phospholipid repeatability was good ($RSE < 6\%$).

Diard et al., (1994) analyzed the lipid and phospholipid composition of chylomicron remnant-like emulsions obtained from the $60 < Sf < 400$ fraction. They found that about 75% of total lipid mass corresponded to TAG, while 20% consisted of phospholipids. The TAG concentration was almost identical to the one reported previously for native chylomicron elsewhere (Redgrave, et al., 1982; Agren, et al., 2006) but the phospholipid concentration differed by about 80%. Results were more discrepant when compared with those of Sultan et al. (1989), who showed 86.5% for TAG and 7.4% for

phospholipids, also measured in native chylomicron remnants. Redgrave and Maranhao (1985) prepared chylomicron remnant-like particles with a **composition of** 70% of triolein and 25% of phosphatidylcholine, and collected particles with 82-84% of TAG and 13-14% (large particles) and 33% (small particles) of phospholipids. The discrepancies among the results shown by others and the results presented here are probably due to the low precision of the methods employed formerly. In the present study, we used HPLC coupled to a light-scattering detector for the simultaneous determination of chylomicron lipid classes. Using the same method, we have previously shown lipid composition of human chylomicrons collected 4h after a load challenge. 4h-chylomicrons were considered as chylomicron remnants and the phospholipid content was very similar to the reported now (about 30%) (Perona and Ruiz-Gutierrez, 2004).

Since the method was proposed by Redgrave and Maranhao (1985), the lipid composition of the resulting particles has been analyzed by different indirect methods for each lipid class, like enzymatic kits for TAG and cholesterol, the o-phthalaldehyde procedure for cholesterol and phosphorus determination for phospholipids (Redgrave and Maranhao, 1985; Lenzo, et al., 1988; Diard, et al., 1994; De Pascale, et al., 2006). These and other lipid classes can be analyzed very accurately by HPLC simultaneously, usually employing normal stationary phases, among which diol columns, have achieved the best results (Perona and Ruiz-Gutierrez, 2004). We have previously shown that chylomicron and VLDL can be accurately determined by HPLC-ELSD, in terms of repeatability of peak areas and retention times (Perona and Ruiz-Gutierrez, 2004; Cabello-Moruno, et al., 2007). This method also allows the detection and quantification of unexpected compounds. In this regard, it was interesting to note the presence of MAG in all artificial chylomicrons, despite this lipid class not having been included in

the preparation, indicating a certain level of hydrolysis, probably from TAG. Another source of MAG might be contamination from the phospholipid standards, which is very unlikely as the supplier (Sigma-Aldrich, St. Louis, MO, USA) declares a purity of 98%.

Chylomicron remnant-like particles have been employed as carriers of bioactive nutrients (Moore, et al., 2003) or drugs (Moore, et al., 2004) but also as models to assess the effects of dietary oils on processes involved in the metabolism of remnants and the development and progress of atherosclerosis (Napolitano, et al., 2001; De Pascale, et al., 2006). However, it is presently not known whether the fatty acid composition of the TAG added to the starting mixture is conserved in the particles and whether the fatty acid composition is homogeneously distributed among the fractions collected after ultracentrifugation. In the present study, triolein was the only TAG used in the starting mixture, and therefore, the only fatty acid expected in the TAG fraction was oleic acid. Intra- and inter-assay repeatability for oleic acid was extremely good, in terms of RSE, indicating that the preparation method is reliable, at least for this fatty acid. Nevertheless, other fatty acids (myristic, palmitic and stearic acids) were found in the TAG fraction. This, together with the presence of MAG among the lipid classes suggests that hydrolysis and, probably, interesterification might be going on in the preparation process. Repeatability was not so good for phospholipid fatty acids, probably due to the variability of each phospholipid origin. Low-frequency sonication with a direct probe has been used to accelerate base-catalized transesterification of vegetable oils (Chemat, et al., 2004; Armenta, et al., 2007) Therefore, it is plausible that a low degree of transesterification might have occurred in the chylomicron fractions in which the phospholipid content is higher ($20 < Sf < 60$ and $60 < Sf < 400$).

Mean particle size, measured by means of DLS, resulted to be significantly higher in the Sf>400 particles, which are in fact, less dense, more TAG-loaded particles. Particle size is an important determinant of TRL metabolic fate and TRL of different sizes are known to be cleared at different rates. Both chylomicrons and VLDL share the same saturable lipolytic pathway, but despite being in a greater number in the postprandial state, VLDL are much slower cleared from plasma, compared to chylomicrons (Bjorkegren, et al., 1996). Xiang et al., (1999) showed that chylomicrons bind more avidly than VLDL to lipoprotein lipase, which is the enzyme responsible of TAG hydrolysis and the first step in TRL clearance. This is of great importance as it is known that delayed TRL clearance may accelerate the development of atherosclerotic plaques (Proctor and Mamo, 1998). In fact, it has been proposed that delayed intravascular catabolism of chylomicron-like emulsions can be an independent predictor of coronary artery disease (Sposito, et al., 2004). DLS is the most widely employed technique for measuring particle size of emulsions. According to this technique, the range of particle sizes in the Sf>400 fraction was more heterogeneous than in the 60<Sf<400 fraction, showing also a higher area under the curve. Thus, when considering the particle mean diameter, results are more reliable for the 60<Sf<400 fraction than for the Sf>400 one. It has been suggested that DLS measurement at forward angle can be biased toward larger particles (Jans, et al., 2009). However, the bias can be as low as 2% for mean DLS diameter (Merkus, 2009).

Conclusions

Despite artificial chylomicrons being used as a model of chylomicron remnants by a number of authors since the method was first proposed in the 1980s, it **has never been** appropriately evaluated. We have now shown that the methodology is reliable and that the emulsion fractions obtained show a close resemblance to the different physiological lipoprotein fractions that are obtained by gradient ultracentrifugation, including the presence of apolipoproteins, and specifically apo E, which are incorporated when incubated with human serum. Importantly, the method was extremely reliable, in terms of lipid composition and particle size, for the $60 < S_f < 400$ fraction, which is the one chosen to mimic chylomicron remnants, the most atherogenic postprandial lipoproteins. Therefore, we consider that this fraction can be used as a model of chylomicron remnants for metabolic studies *in vitro*. Nevertheless, we consider that the apolipoprotein transfer to artificial chylomicrons needs to be more exhaustively studied.

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Declaration of interest

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