1. Introduction

Generally speaking, food-grade nanoemulsions are oil-in-water emulsions with an oil droplet size (diameter) less than 200 nm. Because of this feature, they have better stability, optical transparency, and larger surface area. As an emerging delivery system, nanoemulsions have been shown to improve the oral bioavailability of hydrophobic bioactives (Hatanaka et al., 2010; Wang et al., 2008). As an important application of nanotechnology in food science, nanoemulsions attract much attention and are expected to have broader application in food science.

On the other hand, the toxicity associated with nanoparticles represents one of the major concerns for nanotechnology. Many nanoparticles, such as carbon nanotubes and metal oxide nanoparticles, have been found toxic in vivo (Bai et al., 2010; Bhabra et al., 2009; Yamashita et al., 2011). However, to our best knowledge, there are so far no studies addressing the toxicity of food-grade nanoemulsions.

Compared with other types of nanoparticles, food-grade nanoemulsions have many unique features, such as safety of the components, digestibility, and exclusively oral administration. All the components in the nanoemulsions are either generally recognised as safe (GRAS) or food additives. The common oil phase, triacylglycerols, and many emulsifiers, such as lecithin, modified starch and proteins, undergo digestion in the gastrointestinal tract. Accordingly, the size, aggregation states and interfacial composition and properties of the oil droplets may change as well (McClements & Rao, 2011; McClements & Xiao, 2012). The exposure organs for food-grade nanoemulsions may be limited to the digestive system. Meanwhile, micron-sized emulsions are widely used in the food industry and accepted by regulatory agencies and consumers.

The small intestine and the liver are two very important organs in the digestive system. During digestion and absorption, nanoemulsions could directly interact with the small intestine epithelium. After absorption in the small intestine, it is possible that intact nanoemulsions could be transported to the liver via the portal vein. Therefore, the possible toxicity of nanoemulsions needs to be examined in these two organs.

Human cancer-derived cell lines can be used to examine the in vitro toxicity (cytotoxicity). Caco-2 cells are derived from human colon cancers. After about a 3-week culture, they differentiate to polarise, generate microvilli on the apical side of the cell membrane and form tight junctions between adjacent cells (Hidalgo, Raub, & Borchardt, 1989). Because of these characteristics, Caco-2 cell monolayers are usually used to mimic the small intestine epithelium, and the permeability of bioactives across Caco-2 cell monolayers is used to predict the absorption in vivo (Hubatsch, Ragnarsson, & Artursson, 2007). Meanwhile, HepG2 cells are derived from human liver cancer and are commonly used...
for in vitro assessment of hepatic toxicity (Bort, Ponsoda, Jover, Gomez-Lechon, & Castell, 1999; Lu & Cederbaum, 2006).

In the present study, the possible cytotoxicity of food-grade nanoemulsions was investigated by using two in vitro cell culture systems and comparing with micron-sized emulsions with the same composition. To mimic the exposure of the small intestine to the nanoemulsions, Caco-2 cell monolayers were treated with nanoemulsions and the cell membrane leakage and tight junction integrity were examined. To investigate the toxicity on the liver, HepG2 cells were treated with nanoemulsions and micron-sized emulsions and the cell proliferation/viability rate were compared. These studies may provide the first set of evidence for the possible toxicity of food-grade nanoemulsions.

2. Materials and methods

2.1. Materials

Medium chain triacylglycerol (MCT) was provided by Stepan Company (NEOBEE 1053). Modified starch was obtained from National Starch (HiCap 100). Tween 20 was purchased from Fisher Scientific. Whey protein isolate (WPI) was from Davisco Foods International (BiPro).

Dulbecco’s Modified Eagle Medium (DMEM), Minimum Essential Medium Eagle (MEM), Hank’s Buffered Salt Solution (HBSS), foetal bovine serum (FBS), 100× non-essential amino acids, 100× penicillin and streptomycin and bovine serum albumin (BSA) were all purchased from Fisher Scientific. Transwell permeable polycarbonate inserts (0.4 μm) were obtained from Corning.

2.2. Preparation of micron-sized and nanoemulsions

Ten grammes of emulsions, consisting of 8.5 g dH₂O, 1.0 g MCT and 0.5 g emulsifiers (modified starch, Tween 20 or WPI), were briefly mixed by magnetic stirring. To prepare micron-sized emulsions, the mixture was homogenised at 6500 rpm for 5 min with a high speed homogenizer (ULTRA–TURRAX T-25 basic, IKA Works). Nanoemulsions with the same composition were generated by ultrasonication at about 175 W for (accumulatively) 5 min. The pH of modified starch-emulsion was acidic (between 4 and 5), and that of WPI and Tween 20 emulsions was nearly neutral. Since the emulsions were greatly diluted in a buffer system during cell treatment, the pH of emulsion did not affect the pH of the cell culture system.

2.3. Measurement of the oil droplet size of emulsions

Photographs of diluted micron-sized emulsions and nanoemulsions were taken with a Nikon TE-2000U inverted microscope. The surface-averaged diameters of micron-sized emulsions were determined by using ImageJ software, to analyse nine photographs. Particle size of nanoemulsions was measured with a DLS-based BIC90 plus particle size analyzer equipped with a Brookhaven BI-9000AT digital correlator (Brookhaven Instrument) at a fixed scattering angle of 90° at ambient temperature.

2.4. Maintenance of Caco-2 and HepG2 cell cultures

Caco-2 cells (passage 35–45) were maintained in DMEM with 10% FBS, 1× non-essential amino acids and 1× penicillin and streptomycin. HepG2 cells (passage 7–17) were cultured in MEM with 10% FBS, 1× penicillin and streptomycin. Cells were incubated at 37°C with 5% CO₂.

2.5. Generation and treatment of Caco-2 cell monolayers

To generate Caco-2 cell monolayers, 0.5 ml of 6 × 10⁵ cell/ml Caco-2 cells was plated onto the inserts (apical compartment) of 12-well plates. Subsequently, 1.5 ml of culture media were added to the lower compartment of each well. Media were changed every two days. Cytotoxicity experiments were performed after 21–29 days of plating.

Before the experiments, Caco-2 cell monolayers were washed with and kept in HBSS (0.5 ml in apical compartment and 1.5 ml in basolateral compartment) for 20–30 min at 37°C before the treatment.

Five microlitres of micron-sized emulsions or nanoemulsions were directly added in the apical compartment to make 1–100 dilution. Then the Caco-2 cell monolayers were kept in a platform shaker set at 100 rpm at 37°C for 2 h.

2.6. Lactate dehydrogenase leakage assay

After treatment of Caco-2 cell monolayers with nano- and micron-sized emulsions, 50 μl of media from the apical chamber were removed and the lactate dehydrogenase (LDH) leakage was determined using a CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit (Promega). One-hundred-time diluted emulsions in HBSS were used as the negative control. Cell lysates of the Caco-2 cell monolayers were used as the positive control. Percentage of relative LDH leakage was calculated as:

\[
\% \text{ relative LDH leakage} = \frac{F_l(\text{treatment}) - F_l(\text{negative control})}{F_l(\text{positive control}) - F_l(\text{negative control})} \times 100\%
\]

where \( F_l \) stands for fluorescence intensity with excitation wavelength at 560 nm and emission wavelength at 590 nm, as described in the kit.

2.7. Measurement of transepithelial electrical resistance (TEER)

Immediately before the emulsion treatment, the TEER across Caco-2 cell monolayers was measured, using the Evmom2 epithelial voltmeter (World Precision Instrument). After treatment, Caco-2 cell monolayers were washed with and incubated in HBSS for 20–30 min, before the post-treatment TEER measurement.

Changes in TEER were expressed as % relative TEER.

\[
\% \text{ relative TEER} = \frac{\text{post-treatment TEER}}{\text{pre-treatment TEER}} \times 100\%
\]

2.8. MTT assay on HepG2 cells

The MTT assays on HepG2 cells were performed using the previously published procedures (Yu & Huang, 2010). Briefly, cells were plated at the density of 1000 cell/well and treated with different dilutions of nanoemulsion or micron-sized emulsions (from 1:100 to 1:1600) for about 24 h, followed by incubation with MTT and UV–Vis absorption measurements.

2.9. Statistics analysis

One-way and two-way analyses of variance (ANOVA) were performed, using SigmaPlot 10.0 with SigmaStat integration (Systat software).
3. Results

3.1. General

In this work, the possible cytotoxicity of nanoemulsions was examined by comparing the cellular response to the treatment of nanoemulsions and micron-sized emulsions, assuming that micron-sized emulsions are not toxic.

3.2. Generation of micron-sized emulsions and nanoemulsions

To investigate the size effect of food-grade emulsions on the cytotoxicity, three micron-sized emulsions and nanoemulsions with the same compositions were prepared by high speed homogenisation and ultrasonication, respectively. Modified starch, Tween 20 and whey protein isolates (WPI) were chosen to represent different types of emulsifiers. As shown in Fig. 1 and Table 1, for all the micron-sized emulsions, the particle size (diameter) of the oil droplets was between 5 and 10 μm. In comparison, the average oil droplet size in the nanoemulsions was less than 200 nm, representing about 50-fold reduction. In Fig. 1, the photographs for the nanoemulsions only show the oil droplets in the “tail” region of the particle size distribution, and the majority of nano-sized oil droplets were not possible to visualise with optical microscopy.

3.3. Examination of the cell membrane integrity on Caco-2 cell monolayers

Caco-2 cell monolayers were treated with micron-sized emulsions and nanoemulsions to mimic the exposure of the small intestine epithelium to the emulsions. After treatment, the cell membrane integrity was examined by detecting LDH leakage (Fig. 2). For untreated Caco-2 cell monolayers, less than 3% of cells had cell membrane rupture throughout the experimental procedures. For the monolayers treated with nanoemulsions and micron-sized emulsions, there were about 3–6% of cells with LDH leakage. From one-way ANOVA, it was revealed that there was no significant difference in the LDH leakage between untreated cells and most of the emulsion treatment, except for the nanoemulsion made with modified starch. More importantly, no significant difference was detected between all three pairs of the micron-sized emulsions and nanoemulsions, suggesting that nanoemulsions did not possess more cytotoxicity on the Caco-2 cell monolayers than did regular micron-sized emulsions.

3.4. Investigation of the tight junction integrity in the Caco-2 cell monolayers

After well differentiation, tight junctions form between adjacent Caco-2 cells, similar to those in the small intestine epithelium.

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Micron-sized emulsion</th>
<th>Nanoemulsion</th>
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<tbody>
<tr>
<td></td>
<td>D_{2,0} (μm)</td>
<td>Diameter (nm)</td>
</tr>
<tr>
<td>Modified starch</td>
<td>10.3 ± 1.0</td>
<td>155 ± 1.4</td>
</tr>
<tr>
<td>Tween 20</td>
<td>5.7 ± 0.8</td>
<td>168.2 ± 0.7</td>
</tr>
<tr>
<td>WPI</td>
<td>8.7 ± 0.9</td>
<td>172.7 ± 1.8</td>
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Data for micron-sized emulsion are presented as means ± standard deviation, n = 9. Data for nanoemulsion are shown as means ± standard error, n = 3.

Fig. 1. Micrographs of micron-sized emulsions and nanoemulsions made with modified starch, Tween 20 and WPI, respectively.
Disruption of the tight junction may make the cell monolayer more permeable to water-soluble (toxic) compounds, which should be avoided in the normal physiological condition. In this study, the TEER values of Caco-2 cell monolayers treated with nanoemulsions were compared to that with micron-sized emulsions and untreated. As shown in Fig. 3, one-way ANOVA revealed that, compared with no treatment, modified starch and WPI emulsions caused TEER decrease, and that Tween 20 emulsion had no apparent effect on the tight junction integrity. These observations are consistent with other groups’ finding that some types of emulsifiers and surfactants may interrupt the tight junctions (Ohno, Nagano, Ogawa, & Muramoto, 2006; Yamashita et al., 2000). More importantly, comparing the three nanoemulsions with the corresponding micron-sized emulsions, respectively, there was no significant difference in terms of the TEER change. With the assumption that regular micron-emulsions are considered safe, nanoemulsions did not display additional disruption of the tight junctions.

Together with the results of the LDH leakage in the previous section, it was illustrated that, compared with micron-sized emulsions, nanoemulsions did not affect the cell membrane and tight junction integrity of Caco-2 cell monolayers. However, nanoemulsions formed by modified starch and WPI were found to affect the proliferation/viability of HepG2 cells. These results suggested that the toxicity of nanoemulsions may be dependent on the emulsifiers, and more in vivo studies are required to examine the hepatotoxicity of nanoemulsions.

3.5. Examination of the cytotoxicity on HepG2 cells

To examine the possible toxicity of the nanoemulsions on the liver, HepG2 cells were used as a model and treated with micron-sized emulsions and nanoemulsions of different dilutions (Fig. 4). From two-way ANOVA, it was revealed that nanoemulsions made with modified starch and WPI caused less cell proliferation/viability than did the corresponding micron-sized emulsions ($p = 0.0105$ and $p = 3.21 \times 10^{-5}$, respectively). On the other hand, Tween 20 nanoemulsion did not affect the cell proliferation/viability more than did its micron-sized counterpart ($p = 0.231$). These results suggested that the toxicity of nanoemulsions may be dependent on the emulsifiers, and more in vivo studies are required to examine the hepatotoxicity of nanoemulsions.

4. Discussion

In this work, three nanoemulsions were prepared and their possible toxicity was examined in vitro. It was found that, compared with micron-sized emulsions, nanoemulsions did not affect the cell membrane and tight junction integrity of Caco-2 cell monolayers. However, nanoemulsions formed by modified starch and WPI were found to affect the proliferation/viability of HepG2 cells.
The aim of this work was focussed on food-grade nanoemulsions. The exposure routes of the nanoemulsions were thus limited to the digestive tract, especially the small intestine, considering its large surface area, and possibly the liver, to where digested and absorbed food are carried for possible metabolism and storage. Based on these considerations, Caco-2 cell monolayers and HepG2 cells were chosen to mimic the small intestine epithelium and the liver hepatocytes, respectively. Meanwhile, micron-sized emulsions were prepared in parallel and served as the control, since it is generally accepted that regular (micron-sized) emulsions do not bear any toxicity to the human body.

In the two experiments on Caco-2 cell monolayers, compared to micron-sized emulsions, nanoemulsions did not reveal apparent toxicity on Caco-2 cell monolayers, suggesting that nanoemulsions may not bear significant toxicity to the small intestine. Actually, it is expected that food-grade nanoemulsions undergo digestion (once ingested) along with other food components and, because of their very large surface areas, nanoemulsions are digested much faster than regular micron-sized emulsions (Li & McClements, 2010; Yu & Huang, 2012). In the in vivo scenario, only a small portion of intact nanoemulsions may have the chance to interact with the small intestine epithelium directly. This interaction, in the mimicked in vitro environment as shown in this study, did not reveal apparent toxicity.

In terms of the hepatic toxicity, it was shown here that modified starch- and WPI-stabilized nanoemulsions affected the proliferation/viability of HepG2 cells, while Tween 20 stabilized nanoemulsions did not reveal significant difference from micron-sized emulsions. The reason for this difference in the emulsifiers is still unknown. One hypothesis is that modified starch and WPI are both (modified) biological molecules and may interact with cell membrane through specific receptors while Tween 20 is synthetic and contains multiple polyethylene glycol (PEG) moieties which may prevent/decrease the interaction with cells, as shown in the case of PEGylated proteins and PEGylated quantum dots nanoparticles (Clift et al., 2008; Tsutsumi et al., 2000). On the other hand, although nanoemulsions formed with modified starch and WPI showed increased cytotoxicity on HepG2 cells, the in vivo implication is still unclear, since there is, so far, no evidence showing that intact food-grade nanoemulsions are able to permeate through the epithelium layers lining the digestive tract and be transported to the liver.

Although in real practice, nanoemulsions are usually used as delivery vehicles for lipophilic compounds, only empty nanoemulsions were used here, considering the possibility that bioactives may act on cells and thus make it hard to interpret the results. To really address the toxicity issue of the nanoemulsion-encapsulated bioactives, in vivo experiments are required and the adverse effect caused by increased oral bioavailability of the bioactives should be carefully examined (Huang, Yu, & Ru, 2010; McClements & Rao, 2011).

5. Conclusions

The possible toxicity of food-grade nanoemulsions was examined in vitro, using Caco-2 cell monolayers and HepG2 cells to mimic the response of the small intestine epithelium and the liver, respectively. It was found that, compared with micron-sized emulsions with the same compositions, nanoemulsions did not reveal significantly more toxicity on Caco-2 cell monolayers and that nanoemulsions made with modified starch and whey protein isolate affected the proliferation/viability of HepG2 cells. These results suggested that nanoemulsions may not affect the small intestine epithelium and that the hepatic toxicity of nanoemulsions may need further investigation in vivo.

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References