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Research Article

In Vitro and *In Vivo* Evaluation of a Water-in-Oil Microemulsion System for Enhanced Peptide Intestinal Delivery

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Abstract. Peptide and protein drugs have become the new generation of therapeutics, yet most of them are only available as injections, and reports on oral local intestinal delivery of peptides and proteins are quite limited. The aim of this work was to develop and evaluate a water-in-oil (w/o) microemulsion system *in vitro* and *in vivo* for local intestinal delivery of water-soluble peptides after oral administration. A fluorescent labeled peptide, 5-(and-6)-carboxytetramethylrhodamine labeled HIV transactivator protein TAT (TAMRA-TAT), was used as a model peptide. Water-in-oil microemulsions consisting of Miglyol 812, Capmul MCM, Tween 80, and water were developed and characterized in terms of appearance, viscosity, conductivity, morphology, and particle size analysis. TAMRA-TAT was loaded and its enzymatic stability was assessed in modified simulated intestinal fluid (MSIF) *in vitro*. In *in vivo* studies, TAMRA-TAT intestinal distribution was evaluated using fluorescence microscopy after TAMRA-TAT microemulsion, TAMRA-TAT solution, and placebo microemulsion were orally gavaged to mice. The half-life of TAMRA-TAT in microemulsion was enhanced nearly three-fold compared to that in the water solution when challenged by MSIF. The treatment with TAMRA-TAT microemulsion after oral administration resulted in greater fluorescence intensity in all intestine sections (duodenum, jejunum, ileum, and colon) compared to TAMRA-TAT solution or placebo microemulsion. The *in vitro* and *in vivo* studies together suggested TAMRA-TAT was better protected in the w/o microemulsion in an enzyme-containing environment, suggesting that the w/o microemulsions developed in this study may serve as a potential delivery vehicle for local intestinal delivery of peptides or proteins after oral administration.

KEY WORDS: intestinal delivery; oral; peptide; water-in-oil microemulsion.

INTRODUCTION

With biotechnological advances, peptide- and protein-based drugs have gained an increasing and irreplaceable share of the pharmaceutical market. However, the majority of peptide and protein pharmaceuticals such as insulin, monoclonal antibody-based drugs, human growth hormone, and interferon-α require administration as injections (1). As a consequence, more widespread applications to treat chronic conditions where the patient can self-medicate are limited. The past decades have seen a great deal of efforts to develop non-parenteral routes of delivery for peptides and proteins. Oral delivery may be the most attractive route yet arguably the most challenging due to poor chemical and physical stability of these molecules. Being polymers of amino acids linked together by peptide bonds with specific three-dimensional conformations (2), most peptides and proteins are hydrophilic with poor membrane permeability, susceptible to proteolytic degradation, and low gastric pH. To overcome these obstacles, many strategies have been applied, such as macromolecular chemical modifications (3–5) and simple and complex formulation approaches (6–8). Among various formulation approaches, microemulsions (2,9) have received considerable attention for the oral delivery of peptides and proteins, especially for enhanced oral absorption (10,11).

Microemulsions are thermodynamically stable dispersions of two immiscible liquids stabilized by surfactants (11),

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and mainly classified into three categories: (a) oil-in-water (o/w), (b) water-in-oil (w/o), and (c) bicontinuous microemulsions. Oil-in-water microemulsions are promising in improving the bioavailability of hydrophobic molecules, including hydrophobic peptides, such as cyclosporine A (12). Water-in-oil microemulsions, on the other hand, have shown potential to improve the oral delivery of hydrophilic peptides or proteins (10,11,13,14). The mechanisms proposed for improved bioavailability using w/o microemulsions were based on increased protection from luminal enzymatic degradation and/or enhancer-induced structural and fluidity changes in the mucosal membrane (13,15,16). The safety evaluation of several orally delivered w/o microemulsions has been investigated (10,11,14), and histological examination showed no intestinal tissue damage or irritation upon acute dosing of the specific vehicles.

However, so far, most investigations of w/o microemulsions for peptide or protein oral delivery have been focused on systemic absorption, while the local intestinal delivery of peptides or proteins for the treatment of local pathologies has been less studied. In this work, the potential of w/o microemulsions for local intestinal delivery of water-soluble peptides after oral administration was explored. The ultimate goal of this work was to develop an oral dosage form to deliver therapeutic peptides locally to the gastrointestinal tract for the treatment of gastrointestinal inflammatory disorders. In gastrointestinal inflammatory disorders, like inflammatory bowel disease (IBD), there is a significant unmet need to deliver immunomodulatory compounds directly to the intestinal mucosa to maximize local concentrations (thereby efficacy) and to minimize systemic toxicity. A novel nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inhibitor peptide, 8K-NBD (KKKKKKKKGG-TALDWSWLQTE), has shown efficacies *in vitro* and *in vivo* to inhibit activated NF-κB, a hallmark of chronic inflammation, but not basal NF-κB activity, and ameliorated intestinal inflammation in experimental IBD models (17). The selective targeting of 8K-NBD to activated NF-κB makes it an excellent therapeutic candidate and our research interest. However, a significant challenge for oral delivery of 8K-NBD is its chemical and biological degradation in the gastrointestinal tract. To address this issue and deliver 8K-NBD or other water-soluble peptides locally to the inflamed intestine, the peptide will be incorporated into w/o microemulsions, which will be followed by incorporating the optimal w/o microemulsion into enteric-coated hard gelatin capsules to further enhance local peptide delivery in the GI where needed. The current work is to test the first part of the hypothesis that w/o microemulsions may provide protection to the peptide incorporated when challenged *in vitro* and *in vivo*, and serve as a viable oral delivery system to enhance intestinal delivery of intact and biologically active water-soluble peptides.

Based on some prior research results (10,11,13,14), w/o microemulsions were developed using several commercially available and pharmaceutically acceptable lipid-based excipients. Miglyol 812 and Capmul MCM are medium-chain (C_8/C_{10}) glycerides, generally recognized as safe, and meet the requirements of the United States Pharmacopeia (USP) and The National Formulary as medium-chain triglycerides and mono- and di-glycerides (18,19), respectively. Tween 80 is a nonionic surfactant included in the Food and Drug Administration Inactive Ingredients Database for oral, IV, IM, topical, and many other preparations (20). Phase diagrams were constructed and microemulsion windows were defined. Representative microemulsions across the microemulsion window of interest were characterized for formulation screening. To test the protective effect of the w/o microemulsions, a fluorescent labeled peptide, 5-(and-6)-carboxytetramethylrhodamine labeled HIV transactivator protein TAT (TAMRA-TAT), which has similar cationic content and cell penetrating properties to 8K-NBD (21), was used as a model peptide. TAMRA-TAT was loaded into a selected w/o microemulsion and evaluated *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials

Miglyol 812 (Caprylic/Capric triglycerides, neutral) was provided by Sasol Germany GmbH (Eatontown, NJ, USA). The fatty acid distribution of Miglyol 812 according to the manufacturer is 57.7% caprylic acid (C_8), 41.6% capric acid (C_{10}), 0.2% caproic acid (C_6), and 0.3% lauric acid (C_{12}). Capmul MCM NF (C_8/C_{10} mono- and di-glycerides) was supplied by Abitec Corporation (Janesville, WI, USA). Capmul MCM contains 53.6% of monoglycerides with 6.3% free glycerol and its fatty acid distribution is 82.6% caprylic acid and 17.4% capric acid. Tween 80 (polyoxyethylene sorbitan monooleate, NF grade) was purchased from Spectrum Chemical MFG. Corporation (Gardena, CA, USA). 5-(and-6)-Carboxytetramethylrhodamine-labeled TAT (47–57) peptide (sequence: TAMRA-YGRKKRRQRRR) was purchased from ANASPEC Inc. (Fremont, CA, USA). 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-dodecanoic acid (BODIPY® FL C_{12}) was purchased from Invitrogen (Eugene, OR, USA). Pancreatin from porcine pancreas (8×USP specifications) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Tissue-Tek Optimal Cutting Temperature (O.C.T.) Compound was supplied by Sakura Finetek USA (Torrance, CA, USA). Fluoro-Gel II, with 4′,6-diamidino-2-phenylindole (DAPI) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Purified deionized water from a Milli-Q Advantage System was used for all of the sample preparation. All other chemicals were of analytical grade.

Microemulsion Preparation and Phase Diagrams

Three pseudoternary phase diagrams were constructed to define the microemulsion windows using the following four components: (a) Miglyol 812, a neutral oil of medium-chain (C_8/C_{10} triglyceride) fatty acid triglyceride, (b) Capmul MCM, a low hydrophilic–lipophilic balance (HLB) surfactant (HLB=5.5–6.0), (c) Tween 80, a high HLB non-ionic surfactant (HLB=15.0), and (d) deionized water. Miglyol 812 and Capmul MCM were combined together at three weight ratios (4:1, 65:22, and 2:1, *w*/*w*). The mixture of Miglyol 812 and Capmul MCM at each weight ratio occupied one apex of the ternary phase diagram, and Tween 80 and water occupied the other two apices, respectively. Samples were prepared by mixing appropriate amounts of Miglyol 812, Capmul MCM, Tween 80, and water in screw-capped

scintillation vials at room temperature by shaking. Hundreds of samples were prepared to define the phase boundaries in each phase diagram. Each sample was further allowed to equilibrate at room temperature for at least 24 h before evaluation, and re-examined after 1 week. The existence of the microemulsion window was identified as the region where clear and transparent formulations were obtained upon visual inspection. All samples were stored at room temperature and the stability of each sample was assessed by visual inspection in terms of clarity over time.

Microemulsion Characterization

Rheological Properties

The shear viscosity of the w/o microemulsions was measured using a Brookfield LVDV-III + Cone and Plate Rheometer (Brookfield Engineering Laboratories, Inc., Middleboro, MA, USA). The temperature was controlled by coupling to a Brookfield TC-500 Refrigerated Bath. Instrument calibration was performed using Brookfield silicone viscosity standards. A CPE 40 spindle was utilized for sample measurements. The shear stress, shear rate, and viscosity of a series of representative microemulsion samples in the microemulsion window of interest were measured. The relationship of shear stress (dynes per square centimeter) *versus* shear rate (per second) was utilized to assess the microemulsion flow behavior.

Photon Correlation Spectroscopy

The droplet diameter of the microemulsion was measured by photon correlation spectroscopy using a Zetasizer Nano ZS (Malvern Instruments Inc., Worcestershire, UK) by backscattering at a fixed angle of 173° at 25°C. All the samples were measured in triplicates without any dilution.

Conductivity Measurements

The conductivity of the microemulsions was measured using YSI 3200 conductivity meter (Yellow Spring Instruments Co. Inc., Yellow Springs, OH, USA) coupled to YSI 3252 dip cell with a cell constant of 1.0 cm^{-1}. The instrument was calibrated using YSI conductivity calibrators. Samples were measured at ambient temperature.

Freeze Fracture Transmission Electron Microscopy (FFTEM)

The morphology of the w/o microemulsion was characterized by examination of freeze fracture replicas using transmission electron microscopy (TEM). A tiny drop of microemulsion was sandwiched between gold double-replica mounts and frozen in liquid nitrogen-cooled Freon. Next, the specimens were fractured in a Balzers BAF 400T freeze fracture device at a stage temperature of −100°C under vacuum. The fractured surfaces were shadowed unidirectionally with evaporated platinum and stabilized by carbon evaporation. The resulting replicas and sample residues were rinsed in distilled water, followed by washing in a solution of 5% sodium dichromate in 50% sulfuric acid. Replicas were then transferred to distilled water, placed onto standard copper microscopy grids, examined, and photographed with a Zeiss EM 900 Transmission Electron Microscope at an accelerating voltage of 60 kV (Carl Zeiss, Thornwood, NY, USA).

Phase Inversion Behavior of W/O Microemulsions

To investigate the phase inversion behavior of w/o microemulsions upon dilution, a lipophilic fluorescent marker BODIPY FL C_{12} was incorporated into a selected w/o microemulsion at the concentration of 0.1% by weight, followed by dilutions of 2-, 5-, 10-, 50-, and 250-fold by weight with water. The w/o microemulsion with BODIPY FL C_{12} before and after dilution were examined under a Nikon Eclipse TE 300 Fluorescence Microscope (Fryer Co. Inc., Huntley, IL, USA). The structure of the diluted sample was further investigated by TEM. The w/o microemulsion after a ten-fold dilution in water was negatively stained as follows: a drop of diluted microemulsion in water was placed on a copper grid, followed by removal of excess sample with filter paper, and then a drop of 2% uranyl acetate was placed on the copper grid for 2 min and subsequently examined by TEM.

To elucidate the phase inversion behavior of samples across the microemulsion window, microemulsions incorporating 5% water and with varying amounts of oil and surfactants were diluted 100-fold in water by weight, and the appearance and particle size of diluted samples were assessed.

In Vitro Stability Studies in Modified Simulated Intestinal Fluid

Modified simulated intestinal fluid (MSIF) was prepared based on USP 34 (*Solutions*/Test Solutions). Briefly, 0.68 g of monobasic potassium phosphate was dissolved in 75 mL of water and mixed with 7.7 mL of 0.2 N sodium hydroxide. Pancreatin (0.125 g) was added and mixed well. The pH was adjusted to 6.8±0.1. Water was added to q.s. to 100 mL, and then 1 mL was taken and diluted with 999 mL phosphate buffer of pH 6.8 (10 mM) to a total of 1,000 mL.

TAMRA-TAT microemulsion was prepared by incorporating a defined amount of the TAMRA-TAT water solution to the mixture of Miglyol 812, Capmul MCM, and Tween 80 to a final composition of Miglyol 812/Capmul MCM/Tween 80/TAMRA-TAT solution at the weight ratio of 62.8/21.2/7/9 with TAMRA-TAT at 60 μg/g of the microemulsion. In the *in vitro* stability studies, 100 μL of MSIF was added to 50 mg of TAMRA-TAT microemulsion or TAMRA-TAT solution (60 μg/g) and incubated at 37°C. At specified time points, the incubation was terminated by adding 300 μL of acetone with 2% (*v*/*v*) of trifluoroacetic acid. The clear fraction containing TAMRA-TAT was taken after centrifugation at 16,000×g for 10 min at 4°C, and then lyophilized and reconstituted in acetonitrile/water with 0.1% trifluoroacetic acid (19/81, *v*/*v*). TAMRA-TAT in the reconstituted sample was determined by high-performance liquid chromatography (HPLC) with fluorescence detection. Briefly, TAMRA-TAT was detected using a reverse-phase column (Vydac 238MS C18 5 μm, 4.6×250 mm, Grace Davison, Deerfield, IL, USA) on a Thermo Finnigan Surveyor Plus HPLC system with a FL

Plus detector using a binary gradient elution. Mobile phase A consisted of 0.1% trifluoroacetic acid in acetonitrile and phase B consisted of 0.1% trifluoroacetic acid in deionized water. The initial eluent contained 19% phase A and 81% phase B, and then phase A was increased to 37% in 14.5 min. The eluent composition was held at 37% phase A for 1 min and then changed back to the initial composition and equilibrated for 5 min before the next injection. The flow rate was kept constant at 1 mL/min. The sample injection volume was 10 μL and the excitation and emission wavelengths were 545 and 579 nm, respectively.

In Vivo Studies for Intestinal Delivery

C57/BL6 female mice were obtained from the Charles River Labs and maintained in specific pathogen-free conditions. All experiments involving mice were carried out according to the protocol approved by the UNC Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill. Twelve-week-old C57/BL6 female mice (18–20 g) were fed with standard chow without fasting and grouped randomly and treated with TAMRA-TAT microemulsion (2.5 mg/kg) or TAMRA-TAT solution (2.5 mg/kg) or placebo microemulsion. TAMRA-TAT microemulsion and TAMRA-TAT solution were prepared as described above with TAMRA-TAT final concentration at 250 μg/g. Placebo microemulsion was prepared in the same manner but using water as the aqueous phase instead of TAMRA-TAT stock. All treatments were administered by oral gavage with a single dose of 200 μL. Mice were sacrificed 4 h later and intestinal tissue was harvested. Intestinal samples from duodenum, jejunum, ileum, and colon were snap frozen in O.C.T. Compound and cut into 7-μm-thick sections and placed onto microscope slides. Tissue on the slides was counterstained with DAPI and examined using fluorescence microscopy on an Olympus I×70 Fluorescence Microscope (Olympus America). Fluorescence intensity (FI) was quantified using ImageJ and expressed as integrated density over the total field of each picture, the same region of interest (ROI). The background was subtracted from each picture by setting up a threshold value. Data are presented as mean±SD. The statistical differences among the three treatments (TAMRA-TAT microemulsion, TAMRA-TAT solution, and placebo microemulsion) and different intestinal sections (duodenum, jejunum, ileum, and colon) were evaluated by linear regression. The statistical significance between the treatments for each intestinal section was assessed by one-way ANOVA with Bonferroni post-tests. A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phase Diagrams

The partial pseudoternary phase diagrams of Miglyol 812/Capmul MCM/Tween 80/water system were constructed and are presented in Fig. 1. Miglyol 812 was the oil phase, Tween 80 was the surfactant, Capmul MCM was the co-surfactant, and water was the aqueous phase. Capmul MCM has an HLB value of 5.5–6.0. It was utilized since it has the same carbon chain length as Miglyol 812. Tween 80 is a water-soluble surfactant with an HLB value of 15. The combination of Capmul MCM and Tween 80 created a more flexible water/oil interface, which in turn stabilized the w/o microemulsions with an overall HLB value in the range from 7 to 9. The weight ratio of Miglyol 812 to Capmul MCM was kept constant at 4:1, 65:22, and 2:1 (*w*/*w*), respectively. The advantage for keeping the weight ratio of Miglyol 812 to Capmul MCM constant was that when the sample composition changed, the sample HLB value would change according to the Tween 80 to Capmul MCM ratio in each phase diagram. As described in the "Materials and Methods" section, the microemulsion window was defined as the region where formation of clear and transparent formulations upon visual examination occurs. During the visual examination process, the flow property of the samples was also considered when the Tween 80 concentration was higher than 50% by weight. Samples that were very viscous and unable to flow when the vial was turned upside down were excluded from the microemulsion window in this study. When comparing the area obtained from these three phase diagrams, the apparent microemulsion window was larger when the oil to co-surfactant ratio was lower. However, during the formulation screening process, a stable system with lower amount of surfactants and similar water incorporation capacity was preferred due to potential lower cytotoxicity (22). Meanwhile, since a lower viscosity was also preferred and microemulsion viscosity increases with the increase of Tween 80 concentration, the weight ratio of Miglyol 812/Capmul MCM at 65:22 (*w*/*w*) was selected in this study. With this ratio, microemulsions with the Tween 80 concentration at 10% by weight could incorporate up to around 9% water by weight. Clear samples inside the microemulsion window were stored at room temperature and monitored over time to assess whether precipitation, phase separation, or any clarity change occurred. All clear samples remained clear for at least 6 months at room temperature, which clearly demonstrated the excellent stability of the optimized systems.

A similar w/o microemulsion system has been reported by Constantinides and colleagues (11,23,24) for systemic peptide delivery. They reported greatly enhanced bioavailability of Calcein and a water-soluble RGD peptide using the system of Captex 355/Capmul MCM/Tween 80/Aqueous (65/22/10/3, in percent *w*/*w*). In the current studies, Miglyol 812, which has more defined fatty acid distribution and different product specifications compared to Captex 355, served as the oil phase and up to 9% water was incorporated in the w/o microemulsions. The feasibility of these formulations for local intestinal delivery of peptides via oral administration was investigated both *in vitro* and *in vivo*.

Characterization of W/O Microemulsions

Rheological Properties and Conductivity

The rheological properties of microemulsion samples in the microemulsion window were characterized by measuring their shear stress *versus* shear rate. Several representative samples across the microemulsion window incorporating 5% water and varying amounts of Tween 80 (Fig. 2a) were measured. As shown in Fig. 2b, all of the 11 microemulsion samples showed apparent Newtonian fluid behavior wherein

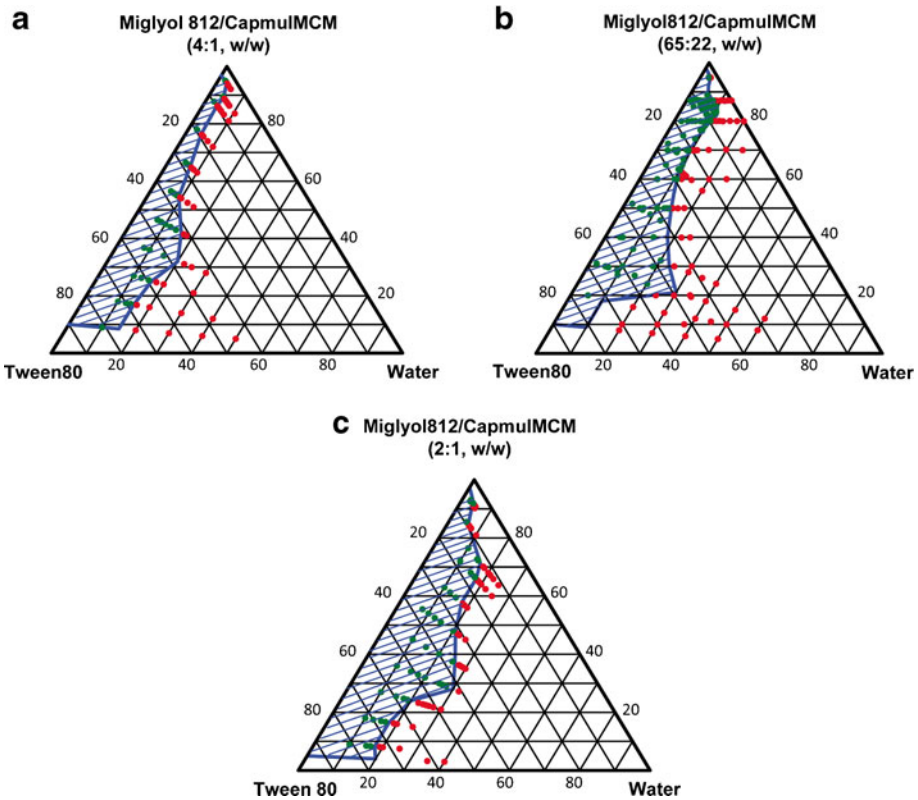

Fig. 1. Partial pseudo-ternary phase diagrams of the system comprising Miglyol 812/Capmul MCM/Tween 80/Water for the development of w/o microemulsions. The weight ratio of Miglyol 812/Capmul MCM was kept constant at **a** 4:1, **b** 65:22, and **c** 2:1, respectively. The *green dots* represent clear samples, the *red dots* represent turbid samples, and the *shaded area* is the microemulsion window

there was a linear relationship between shear stress and shear rate. The viscosity of the 11 samples was obtained by linear regression, and the slope of each regression line was the apparent viscosity of each sample. As shown in Fig. 2c, the viscosity of the microemulsion samples increased with an increase in Tween 80 concentration. The viscosity data also indicated the presence of a threshold (at the line around 42% Tween 80). When the Tween 80 concentration was below this threshold, the effect of Tween 80 on the microemulsion viscosity was minor; however, when the Tween 80 concentration was above this threshold, the effect of Tween 80 on the microemulsion viscosity was more pronounced. This phenomenon indicated that there might be a microemulsion structure change when the Tween 80 concentration increased up to around 42% by weight (Fig. 2c), which was consistent with the results of the dilution studies discussed later. The detailed microstructure is not yet understood. We hypothesize that below this threshold, the w/o microemulsion droplets and micelles coexisted, and the enhanced viscosity was due to the increase of micelle number and size. Moreover, above this threshold, there was the existence of a bicontinuous microemulsion where Tween 80 and Miglyol 812 existed as two continuous phases with Capmul MCM dispersed in the interfacial layer and water dispersed in the continuous Tween 80 phase or associated with the polar head group of Tween 80 in the interfacial layer. Thus, the viscosity increase above this threshold was attributed to the increase of bulk Tween 80.

To elucidate the sample properties inside the microemulsion window, a series of microemulsion samples at different ratio of Miglyol 812/Capmul MCM (65:22, *w*/*w*) to

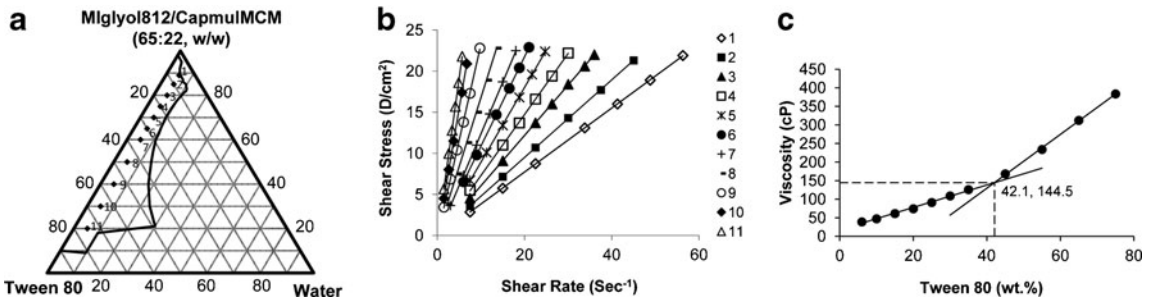

Fig. 2. The mapping **a**, rheological property **b**, and viscosity **c** of 11 microemulsion samples containing 5% water inside the microemulsion window for characterization. The sample numbers on the legend of **b** correspond to the samples shown in **a**

Tween 80, such as 85:10, 70:25, 50:45, and 30:65, were prepared and characterized. The viscosity of these microemulsion samples is shown in Fig. 3a. At each fixed oil to surfactant ratio, the viscosity increased slowly with increasing water content. This increase was most likely due to the increase in both droplets number and size which enhanced the dispersed phase droplet interaction. In addition, consistent with the results above, as the Tween 80 concentration increased, the viscosity increased accordingly.

Conductivity is an important parameter which might reflect the microstructure of the microemulsions (25). The conductivity of series of microemulsion samples with different oil to surfactant ratios is shown in Fig. 3b. With increasing water concentration at each fixed oil to surfactant ratio, the conductivity increased accordingly, most likely due to the increase in both droplets number and size (26). Related, with increasing Tween 80 concentration, the conductivity also gradually increased, which was probably due to the increased interaction between droplets which facilitated the ion exchange between droplets (26). Overall, the low conductivity of these microemulsions was an indication of the formation of w/o microemulsions (27).

Photon Correlation Spectroscopy and FFTEM

Photon correlation spectroscopy was employed for microemulsion droplet size analysis. As shown in Fig. 3c, samples in the microemulsion window with water concentration higher than 3% and Tween 80 concentration lower than 20% showed smaller polydispersity index (PI) with more uniform droplet diameter in the range of 10–40 nm (data not shown). While samples in the microemulsion window with Tween 80 concentration at 20% (*w*/*w*) or higher, or with water concentration lower than 3% (*w*/*w*) were polydisperse with a greater PI. The size analysis reports indicated that those samples with a larger PI (e.g., PI>0.3) were too polydisperse for data analysis. The high PI values of the microemulsion samples were likely an indication of the presence of an excess amount of Tween 80 surfactant. Since samples with uniform size distribution were preferred, microemulsion samples with smaller PI (PI<0.1) were identified for further evaluation.

To investigate the morphology and confirm the droplet size of the w/o microemulsions, FFTEM was utilized. Figure 4 shows the FFTEM results of one of the placebo microemulsion formulations of interest (Table I), which was plotted and highlighted by a red circle and a red arrow in Fig. 3c. The droplets were spherical with an average diameter around 20 nm, which was consistent with the results obtained by photon correlation spectroscopy.

Phase Inversion Behavior of W/O Microemulsions

The w/o microemulsions developed in this work were primarily designed for oral delivery route. After oral administration, the w/o microemulsions will likely be phase-inverted into o/w emulsions and/or water-in-oil-in-water (w/o/w) double emulsions. Since the phase inversion behavior might affect the *in vivo* fate of the drug incorporated (28), it is important to investigate this behavior. The first experiment performed was a dilution study using fluorescence microscopy. A fluorescent fatty acid, BODIPY FL C_{12}, was dissolved in a w/o microemulsion formulation (84% mixture of Miglyol 812/Capmul MCM (65:22, *w*/*w*), 10% Tween 80, and 6% water), and then diluted in water by different dilution factors. The purpose of this dilution study was, (1) to confirm the phase inversion behavior and (2) to find out the minimal dilution factor required for the phase inversion to occur. As expected, before dilution, the microemulsion with BODIPY FL C_{12} appeared clear with strong green fluorescence

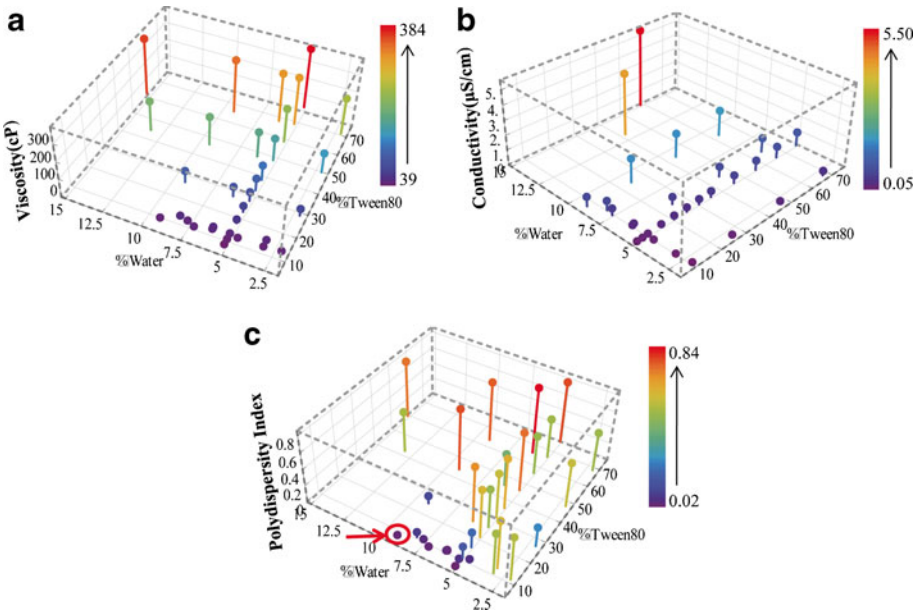

Fig. 3. The viscosity **a**, conductivity **b**, and polydispersity index **c** of microemulsion samples across the microemulsion window with the weight ratio of Miglyol 812/Capmul MCM at 65:22 (*w*/*w*). Each data point is color coded and the height of each bar also indicates the relative value of each data. The *circled* data point with a *red arrow* corresponds to the placebo microemulsion shown in Table I

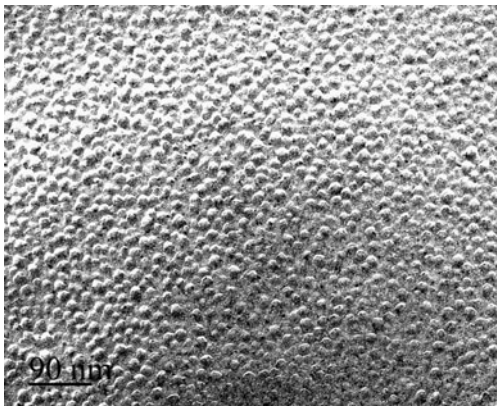

Fig. 4. The FFTEM of a selected placebo water-in-oil (w/o) microemulsion

(Fig. 5a). However, upon a five-fold or more dilution, the w/o microemulsion was readily inverted into coarse o/w-type emulsions with large and polydisperse green fluorescent droplets (Fig. 5c–f), while with a two-fold dilution, the w/o microemulsion was probably partially phase-inverted as a large continuous green fluorescent area occupied close to half of the examined field (Fig. 5b). This study visually demonstrated the occurrence of phase-inversion behavior of w/o microemulsions upon dilution.

To further examine the detailed structure of the resulting emulsion from dilution, a w/o microemulsion formulation (84% mixture of Miglyol 812/Capmul MCM (65:22, *w*/*w*), 10% Tween 80, and 6% water) diluted ten-fold by weight in water was negatively stained using 2% uranyl acetate, and subsequently examined using TEM. The results showed that the large droplets seen under a fluorescence microscope contained numerous smaller droplets associated together (Fig. 6), and there was no clear indication of the formation of double or multiple emulsions. The dark area between those smaller droplets might be an indication of the presence of aqueous phase, which may be able to host water-soluble drug molecules and protect the drug molecules from acidic and enzymatic degradation. The oil phase might also be able to bring the peptide to the proximity of mucosa surface quicker so that the peptide has a greater chance to be internalized before degradation occurs in the lumen. The phase inversion studies using fluorescence microscopy together with TEM provide a high resolution perspective of phase inversion from w/o microemulsions to coarse o/w emulsions upon dilution using aqueous phase.

To provide an overview of the phase inversion behavior of samples across the microemulsion window, samples with 5% water and different oil to surfactant ratios were diluted 100-fold in water by weight. The results showed that as the weight ratio of Miglyol 812/Capmul MCM (65:22, *w*/*w*) to Tween 80 was 40:55 (#7 in Fig. 7a) or less, such as 40:60, 30:65, 20:75, 10:90 (corresponding to #8, 9, 10, and 11 in Fig. 7a), the microemulsion was inverted to stable clear or translucent microemulsion (Fig. 7a) with uniform size distribution (Fig. 7b). However, when the weight ratio of Miglyol 812/Capmul MCM (65:22, *w*/*w*) to Tween 80 was 45:50 (#6 in Fig. 7a) or higher, the microemulsion was inverted to turbid emulsions. These results were consistent with the viscosity data which showed the existence of a threshold around the line of 42% Tween 80 by weight. Together, the viscosity data and dilution data indicated there might be a microstructure change of microemulsions with Tween 80 concentration close to 42–50% by weight in the microemulsion window of the selected phase diagram. In addition, since the microemulsion with the weight ratio of Miglyol 812/Capmul MCM (65:22, *w*/*w*) to Tween 80 at 40:55 (#7 in Fig. 7a) or lower exhibited similar dilution properties of self-microemulsifying drug delivery systems (29,30), these microemulsions might find applications to enhance oral absorption of poorly water-soluble and/or poorly permeable drugs, which is beyond the scope of the current study.

In Vitro Stability Studies in MSIF

MSIF was prepared using pancreatin from porcine pancreas, which contains a broad spectrum of digestive enzymes, including amylase, trypsin, lipase, ribonuclease, and protease. The pancreatin from porcine pancreas contains similar enzymes as the pancreatic fluid which a drug might encounter when it enters the intestines following oral administration. To test the hypothesis that w/o microemulsion can provide protection to the water-soluble peptide incorporated from enzymatic degradation, the stability of TAMRA-TAT in microemulsion and TAMRA-TAT in solution in MSIF at 37°C was determined by HPLC with fluorescence detection. The TAMRA-TAT peak in HPLC chromatogram was well separated from its degradation products. Spike-recovery studies of TAMRA-TAT in the presence of placebo microemulsion using the sample processing method described under "Materials and Methods" section was 100.11±6.24% (n=3). The kinetics of TAMRA-TAT degradation was determined by following the loss of TAMRA-TAT as a function of time (31). As shown in Fig. 8, the kinetics of TAMRA-TAT microemulsion and solution in MSIF followed pseudo-first-order rates, which were described by the equation $y=b\times e^{-kt}$. In this equation, y represents the percentage (normalized concentration) of TAMRA-TAT at time t, b is the initial TAMRA-TAT concentration (percentage), t is the reaction time in minutes, and k is the pseudo-first-order rate constant (31). For TAMRA-TAT solution, the TAMRA-TAT degradation kinetics fit the equation $y=96.78\ e^{-0.1034t}$ (R^2= 0.9977), and the half-life ($t_{1/2}$) calculated from the resulting

Table I. Characterization of Placebo and TAMRA-TAT W/O Microemulsions (n=3)

w/o Microemulsions	Viscosity(cP)	Mean droplet diameter (nm)	Polydispersity index	Conductivity (μS/cm)
Placebo ME	43.7±1.2	20.6±2.3	0.023±0.030	0.690±0.013
TAMRA-TAT ME	43.2±0.6	21.4±3.3	0.059±0.036	0.681±0.017

This w/o microemulsion composition was Miglyol 812/Capmul MCM/Tween 80/Aqueous at the weight ratio of 62.8/21.2/7/9. The aqueous for placebo microemulsion (ME) is water and for TAMRA-TAT microemulsion is TAMRA-TAT stock

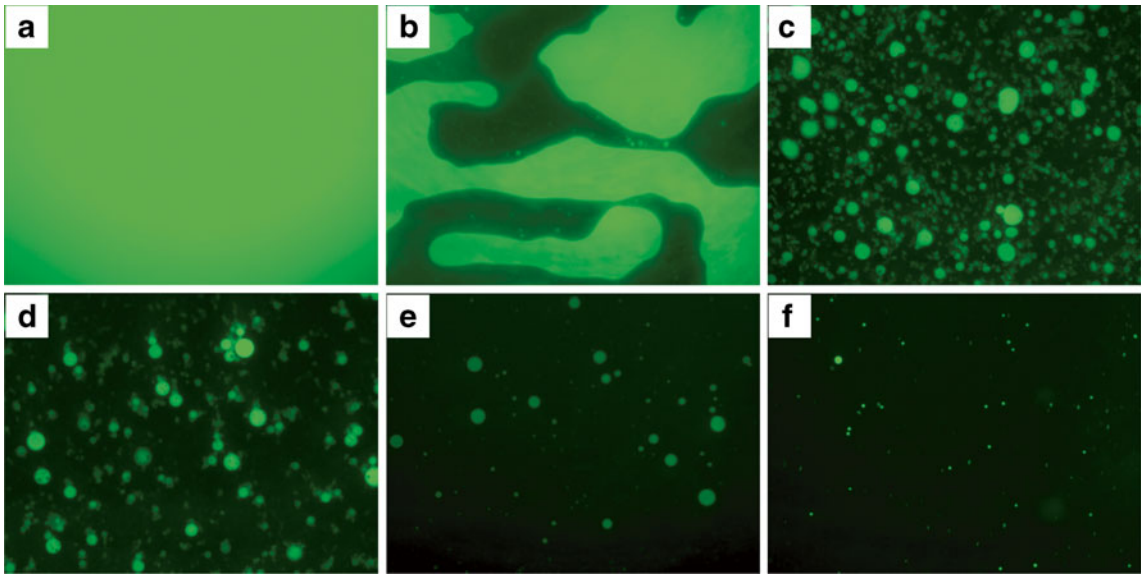

Fig. 5. Fluorescence microscopy of water-in-oil (w/o) microemulsion upon dilution in DI water. **a** W/O microemulsion with BODIPY FL C_{12}, **b** w/o microemulsion with BODIPY FL C_{12} by a two-fold dilution, **c** five-fold dilution, **d** ten-fold dilution, **e** 50-fold dilution, and **f** 250-fold dilution, respectively. The composition of the w/o microemulsion was Miglyol 812/Capmul MCM/Tween 80/water at the weight ratio of 62.8/21.2/10/6. All the images shown were taken with ×10 objective lens (with a total of ×100 magnification)

rate constant was 6.7 min. For TAMRA-TAT microemulsion, the TAMRA-TAT kinetics followed $y=91.56\ e^{-0.0357t}$ ($R^2=0.991$) with a calculated $t_{1/2}$ of 19.4 min, which was nearly three-fold greater than that of TAMRA-TAT in solution.

The HIV TAT (47–57) moiety in TAMRA-TAT contains positively-charged amino acids arginine and lysine, which makes it highly susceptible to proteolysis by tryptic enzymes (31). A review article (32) pointed out seven potential trypsin cleavage sites (48, 49, 50, 51, 52, 54, and 56), and Grunwald *et al.* (31) also identified a few fragments by MS in *in vitro* TAT (47–57) proteolysis studies, which include GRKKRRQRRR, GRKKRRQRR, GRKKRRQR, YGRKKRRQR, and YGRKKRRQRR. In our study, the kinetics of TAMRA-TAT degradation was followed by the detection of the loss of parent TAMRA-TAT. The stability of the TAMRA-TAT was significantly enhanced by the w/o microemulsion when challenged by MSIF. A similar protective effect from a different w/o microemulsion system to a rhPTH1-34 peptide in the presence of pepsin and trypsin was reported by Guo *et al.* (33) in an *in vitro* enzymatic stability study. Another study by Cheng *et al.* (10) stated that after their w/o microemulsion system was phase inverted following a 800-fold dilution, only 7% loaded peptide was detected in the aqueous phase and the rest of the peptide was still detected in the upper coarse emulsion after separation by centrifugation. Although, there was no direct evidence, it is possible that even after dilution,

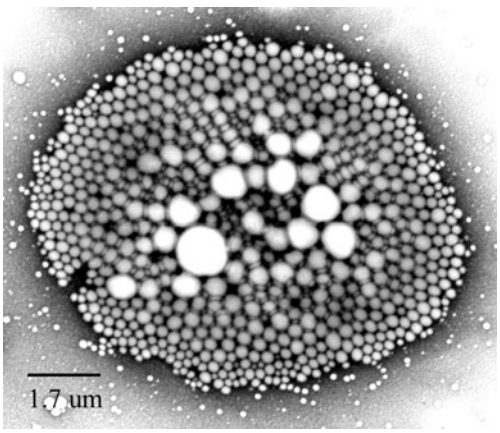

Fig. 6. TEM of a w/o microemulsion sample after a ten-fold dilution. The w/o microemulsion composition was the same as stated under Fig. 5.

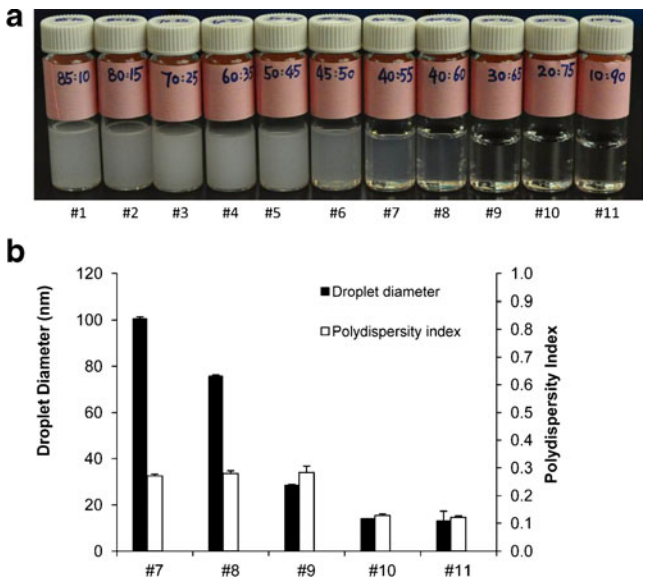

Fig. 7. The phase inversion behavior of w/o microemulsions. **a** The picture shows the phase inversion behavior of samples across the microemulsion window. Eleven microemulsion samples with the weight ratio of Miglyol 812/Capmul MCM (65:22, *w*/*w*) to Tween 80 at 85:10, 80:15, 70:25, 60:35, 50:45, 45:50, 40;55, 40:60, 30:65, 20:75, and 10:90 were diluted 100-fold by weight with water, respectively. **b** The droplet size analysis of diluted samples corresponds to samples labeled in **a**

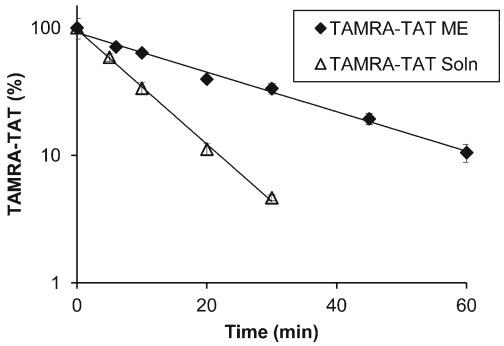

Fig. 8. The stability of TAMRA-TAT microemulsion *versus* TAMRA-TAT solution (*Soln.*) in MSIF at 37°C. The remaining TAMRA-TAT was normalized with respect to the initial concentration. Three samples were prepared for each time points for both TAMRA-TAT microemulsion and TAMRA-TAT solution. Data are presented as mean±SD (n=3) on a logarithmic scale. The degradation profiles of TAMRA-TAT in microemulsion and solution in MSIF at 37°C were described by pseudo-first-order kinetics

the peptide loaded to the microemulsion might still be associated with the coarse emulsion when phase inversion happens, which renders protection to the loaded peptide from enzymatic degradation. These peptide stability data further supported the results from the following *in vivo* mouse studies and the hypothesis that w/o microemulsion may provide protection to the peptide incorporated in the enzymatic environment compared to peptide in water solution.

In Vivo Studies for Intestinal Delivery

The initial aim of this work was to develop and optimize a w/o microemulsion that has the potential to increase the delivery of a potent peptide to inflamed intestinal tissue. If successful, the w/o microemulsion could then be incorporated into enteric-coated hard gelatin capsules to further enhance the delivery where and when needed. Therefore, it was not the intention to develop a w/o microemulsion that would be given orally as an oral microemulsion drink. To facilitate the initial aim, a commercially available fluorescent TAMRA-TAT peptide was used as a model therapeutic peptide, considering it has similar cationic content and cell-penetrating properties to 8K-NBD, a potent NF-κB inhibitor. TAMRA-TAT was loaded to one of the w/o microemulsion templates (Table I) and orally gavaged to mice. TAMRA-TAT solution and placebo microemulsion were administered in parallel as controls. The physical properties of the w/o microemulsion with and without TAMRA-TAT are shown in Table I. The actual TAMRA-TAT loading was 96.6±1.69% (n=5) compared to the theoretical loading as measured by HPLC.

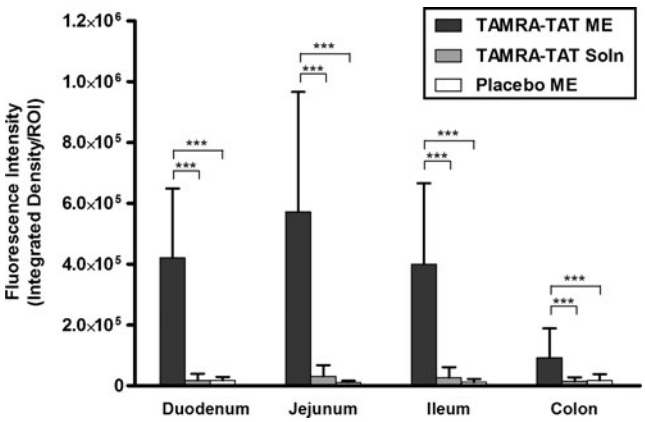

Fig. 9. The fluorescence intensity in different mouse intestinal sections after oral administration of TAMRA-TAT microemulsion (n=4 mice), TAMRA-TAT solution (Soln.; n=4 mice), and placebo microemulsion (n=3 mice). The data are presented as mean±SD. ***p<0.001

The intestinal distribution of TAMRA-TAT was evaluated using a semi-quantitative method, fluorescence microscopy. The FI was quantified using ImageJ and represented as integrated density over the same ROI. As shown in Fig. 9, the FI from the treatment of TAMRA-TAT microemulsion in all the intestinal sections are significantly greater than that from the treatment of TAMRA-TAT solution or placebo microemulsion. Linear regression of FI *versus* treatments (TAMRA-TAT microemulsion, TAMRA-TAT solution, and placebo microemulsion) and FI *versus* intestinal sections (duodenum, jejunum, ileum, and colon) showed p values of 1.51×10^{-26} and 3.52×10^{-6}, respectively, which indicated FI was strongly correlated with the treatments, meanwhile, FI was associated with intestinal sections, but to a lesser extent. To compare the overall FI in the small intestines to that in the large intestine, the linear regression coefficients of FI *versus* treatments in each intestinal section were calculated. The average linear regression coefficient from the duodenum, jejunum, and ileum was 5.9-fold greater than that from the colon, which suggested that overall, the FI difference among three treatments in the small intestine was greater than that in the large intestine. Representative images from each intestinal section are shown in Fig. 10.

In summary, the *in vivo* mouse experiment showed that FI resulting from the TAMRA-TAT microemulsion treatment was significantly greater than that from the TAMRA-TAT solution or placebo microemulsion treatment in all intestinal sections. Although a semi-quantitative method was employed in these *in vivo* studies, it was likely that the fluorescence observed was mostly due to intracellular fluorescence since the intestine tissue was washed before fluorescence imaging. Moreover, only intact TAMRA-TAT should be taken up intracellularly, so the presence of more fluorescence in the tissue was an indication of the presence of more intracellular TAMRA-TAT. Therefore, results from the *in vivo* studies showed that the TAMRA-TAT w/o microemulsion enhanced the intestinal delivery of TAMRA-TAT compared to the TAMRA-TAT solution and placebo microemulsion. However, the overall FI in the small intestine was still greater than that in the large intestine after oral administration, which will be addressed by encapsulation of the w/o microemulsion into enteric-coated capsules to facilitate the local delivery to the target region of the intestinal tract, such as the colon. Some preliminary experiments on the compatibility of the w/o microemulsion and hard gelatin capsules were carried out. The results showed that hard gelatin capsules containing Miglyol 812, Capmul MCM, Tween 80, and the w/o microemulsion, respectively, remained stable at room temperature for at least 1 year. There were no obvious changes to capsule stiffness or shape. The w/o microemulsion inside the hard gelatin capsules stayed clear with the same particle size after 1 year at room temperature. The compatibility of the excipients in w/o microemulsions contained in hard gelatin capsules was also

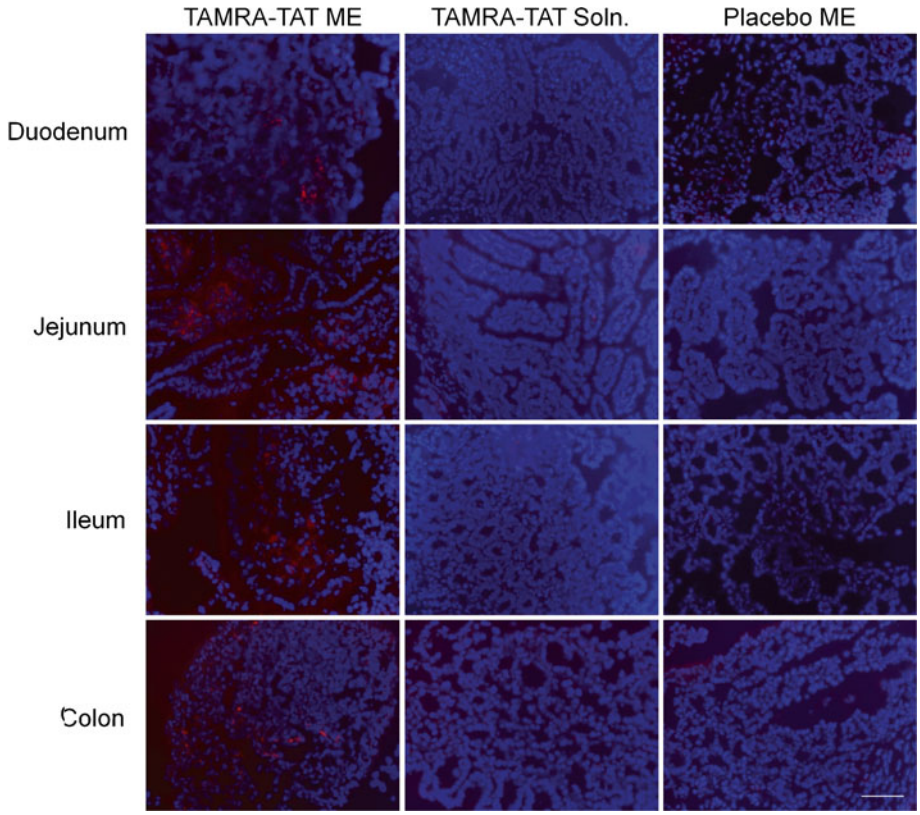

Fig. 10. Representative fluorescence microscopic images from intestines of C57/BL6 mice treated with TAMRA-TAT microemulsion, TAMRA-TAT solution (Soln.) and placebo microemulsion (ME) 4 h after oral gavage. *Scale bar* represents 50 μm

supported by a review (34). After capsules containing w/o microemulsion were enteric coated, the microemulsion stayed clear and stable in enteric-coated capsules at room temperature or at 4°C in a refrigerator for at least 1 week before being used for experiments. Results from the mouse experiments were also supported by the data from the TAMRA-TAT *in vitro* stability studies, which provided direct evidence on the protective effect of w/o microemulsion to the peptide incorporated when challenged by the enzyme-containing environment. Together, the *in vitro* and *in vivo* studies indicated that the w/o microemulsion system may serve as a viable oral delivery vehicle to enhance the intestinal delivery of water-soluble peptides.

Intestinal delivery of peptides and proteins has been reported (35), but most of the reports have focused on the improvement of systemic absorption. There are quite limited reports on the local intestinal delivery of peptides or proteins for the treatment of local pathologies, like IBDs. Coco *et al.* (36) investigated the potential of three polymeric nanoparticles for colon-specific protein delivery via *in vitro* and *ex vivo* approaches. Hong *et al.* (37) reported promising peptide local intestinal delivery using colon-targeted enteric-coated capsules. In our study, w/o microemulsions as a potential oral delivery vehicle for local intestinal delivery of a cell-permeable peptide was investigated. The hydrophobic oil phase, Miglyol 812, together with Capmul MCM served as a protective vehicle to shield the peptide from acidic and enzymatic attack and protect the peptide incorporated during transition. In addition, Miglyol 812 as medium-chain triglycerides might also be converted to monoglycerides in the presence of lipase and enhance intracellular delivery (38). In our study, the protective effect of the w/o microemulsion to the peptide incorporated was clearly presented. Incorporation of therapeutic peptides into the w/o microemulsion developed in the current study followed by encapsulation into enteric-coated capsules will be evaluated in larger animal models, like pig colitis models and will be the focus of our future studies.

CONCLUSIONS

This work demonstrates the potential of using w/o microemulsions as an oral delivery vehicle to deliver a cell-permeable peptide to the local murine intestines. Water-in-oil microemulsions were developed, characterized, and evaluated. The *in vitro* peptide stability studies provided direct evidence of the protective effect of the w/o microemulsion to the peptide incorporated against enzymatic degradation. The *in vivo* mouse studies exhibited enhanced intestinal delivery of the model peptide TAMRA-TAT to the mouse small and large intestines using the w/o microemulsion system. Since the w/o microemulsions developed in this work might exhibit dual mechanisms by providing both enhanced protection and intracellular delivery, their applications are not limited to cell-penetrating peptides. Meanwhile, the w/o microemulsions developed in this work were thermodynamically stable, and formed spontaneously at room temperature without the application of heating or high shear stress. These microemulsion systems may serve as a platform for oral intestinal delivery of water-soluble and heat-labile peptides or proteins by their incorporation into enteric-coated capsules.

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