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## Development and characterization of resveratrol nanoemulsions carrying dual-imaging agents

**Aim:** Delivery of the natural anti-inflammatory compound resveratrol with nanoemulsions can dramatically improve its tissue targeting, bioavailability and efficacy. Current assessment of resveratrol delivery efficacy is limited to indirect pharmacological measures. Molecular imaging solves this problem. **Results/methodology:** Nanoemulsions containing two complementary imaging agents, near-infrared dye and perfluoropolyether (PFPE), were developed and evaluated. Nanoemulsion effects on macrophage uptake, toxicity and NO production were also evaluated. The presence of PFPE did not affect nanoemulsion size, zeta potential, colloidal stability, drug loading or drug release. **Conclusion:** PFPE nanoemulsions can be used in future studies to evaluate nanoemulsion biodistribution without interfering with resveratrol delivery and pharmacological outcomes. Developed nanoemulsions show promise as a versatile treatment strategy for cancer and other inflammatory diseases.

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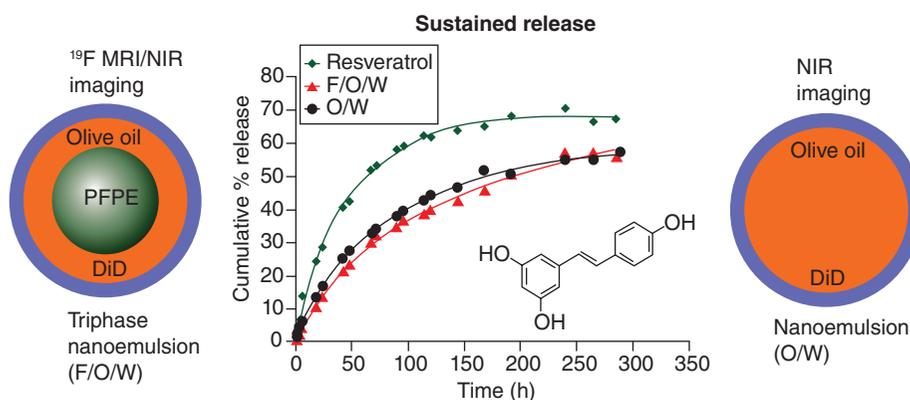
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Resveratrol, a natural antioxidant found in grape skin, red wine and berries, demonstrates anti-inflammatory [1], anticancer [1] and neuroprotective [2] effects. Specifically, resveratrol has been shown to inhibit the

release of proinflammatory mediators from activated macrophages [3–5]. However, when consumed through dietary sources, resveratrol clinical efficacy results vary between studies and seem to be confounded by metabolite

pharmacological effects, which also vary across different disease states [6]. Mixed clinical data may also be in part due to resveratrol's poor bioavailability, poor water solubility and chemical instability [7].

One method of improving the bioavailability and chemical instability of poorly water soluble drugs such as resveratrol is through encapsulation of the drug into a nanoemulsion. Nanoemulsions are kinetically stabilized emulsions with low surfactant concentration (<10% w/v) that are typically produced using high energy methods such as microfluidization or sonication. Several groups have recently reported the incorporation of resveratrol into oil in water nanoemulsions [7–16]. The encapsulation of resveratrol in nanoemulsions has been demonstrated to be an effective method of increasing its bioavailability [9,13]. Nanoemulsions have also been shown to protect resveratrol against chemical degradation and prevent its isomerization to inactive *Z*-resveratrol [8].

All resveratrol-loaded nanoemulsions reported to date are formulated as two-phase systems (oil in water) that do not contain imaging agents. As such, *in vivo* biodistribution of these nanoemulsions may be difficult to study. For such systems, nanoemulsion efficacy is usually determined through pharmacological testing in cells and animals. Hence it is not easy to directly connect resveratrol delivery to specific tissues and cells with pharmacological outcomes. Molecular imaging agents incorporated into resveratrol nanoemulsions, as reported here, would dramatically increase our understanding of how nanoemulsions improve resveratrol bioavailability and *in vivo* efficacy by providing an imaging signature of their distribution to tissues and cells. Therefore, the first aim of this work was to develop a theranostic resveratrol nanoemulsion. Theranostic nanomedicine treatments such as theranostic nanoemulsions have the potential to address inter- and inpatient variability by simultaneously targeting the disease site and providing imaging feedback regarding treatment efficacy [17,18]. Imaging feedback makes it possible to more rapidly determine whether a treatment is adequately addressing the underlying pathology. This real-time visual feedback can also be used to alter patient dosing throughout the course of treatment, thus providing personalized medical treatment to an individual patient [18,19].

To the best of our knowledge, nanoemulsions reported here are first examples of theranostic resveratrol nanoemulsions. Developed theranostic resveratrol nanoemulsions were designed to passively target macrophages, immune cells known to produce inflammatory mediators such as NO [20], with the final goal of improving the anti-inflammatory and anticancer effects of resveratrol. Developed theranostic resveratrol nanoemulsions contain a single therapeutic agent (resveratrol) and dual diagnostic agents; 1,1'-dioctadecyl-

3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR), a near-infrared (NIR) imaging agent and perfluoropolyether (PFPE), a <sup>19</sup>F MRI reagent. The incorporation of DiR is advantageous because NIR fluorescence imaging enables rapid and inexpensive monitoring of nanoemulsion biodistribution *in vivo*. A potential concern with NIR dyes is their escape from the nanoemulsion. DiR is a well-known, commercially available lipid tracer and was loaded into the nanoemulsion oil phase to levels that assure complete solubilization. In earlier studies we reported extensive optical evaluations of commercial dyes and custom synthesized dyes in perfluorocarbon/hydrocarbon mixed oil nanoemulsions [21]. DiD-loaded nanoemulsions with similar composition to those reported here have been extensively evaluated in previous studies, and it was demonstrated that there was no loss of DiD dye [22–24]. Other potential concerns with NIR fluorescence imaging include limited penetration depth and decreased signal intensity with time. This leads to the need for bimodal imaging and inclusion of a <sup>19</sup>F MRI tracer [25]. PFPE is a biologically and metabolically inert MRI tracer. Thus, nanoemulsions containing PFPE are ideal for long-term follow-up of drug delivery with nanoemulsions *in vivo* [26–28]. Additionally, PFPE biodistribution can be monitored *in vivo* without background interference because organic fluorine is not present in the human body [28–30].

In earlier studies, we demonstrated that NIR fluorescence imaging can be used as a complementary method to <sup>19</sup>F MRI to monitor monocytes labeled with PFPE nanoemulsions in live animals due to high correlation between the two imaging modalities [31]. Delivering resveratrol with PFPE nanoemulsions hence provides a unique advantage over other formulations, as <sup>19</sup>F MRI is already demonstrated as a safe, quantitative imaging technique in humans [32]. Hence, the presence of PFPE will allow for the evaluation of resveratrol nanoemulsion biodistribution in human studies in the future. PFPEs, though highly effective as MRI agents for quantitative live imaging of drug delivery in animals and humans, suffer from certain drawbacks and as drug excipients. PFPEs and all fully fluorinated compounds (perfluorocarbons) demonstrate high lipophobicity and extreme hydrophobicity [27]. These properties may interfere with overall PFPE nanoemulsion performance as a drug delivery agent.

Two main strategies have been reported to improve perfluorocarbon nanoemulsion drug carrying capacity. The first strategy uses phospholipids in the surfactant layer of the nanoemulsion as the main carrier for the drug [33,34], while the second strategy, developed earlier by our group, uses the formulation of triphasic nanoemulsions, where PFPE and hydrocarbon oils are

combined into a nanoemulsion internal phase. In these formulations, the hydrocarbon oil represents the key drug carrying excipient while PFPE is passive in this regard [22,35]. The impact of the incorporation of perfluorocarbons such as PFPE on nanoemulsion colloidal behavior and drug carrying capacity has not been reported. Therefore, the second aim of this work was to evaluate whether incorporation of PFPE influences resveratrol nanoemulsion colloidal or drug delivery properties, such as drug loading and drug release.

Resveratrol nanoemulsions reported here were produced both with and without PFPE. In future clinical translation, after nanoemulsion biodistribution is well understood and an effective dosing regimen is determined for the patient; inclusion of PFPE would no longer be necessary. Therefore, PFPE nanoemulsion could be replaced with its simpler, PFPE-free counterpart. In order for this interchangeability to be possible, the presence of PFPE must not affect nanoemulsion drug loading, drug release or colloidal stability. Developed nanoemulsion formulations were evaluated for size,  $\zeta$ -potential, physical stability, drug loading and *in vitro* release profile. Additionally, nanoemulsions were evaluated for uptake, toxicity and effect on NO production in RAW 264.7 macrophages as a model inflammatory cell line.

## Materials & methods

### Materials

Resveratrol was purchased from Supelco (PA, USA) and used without further purification. Pluronic® P105 was obtained from Basf Corporation (NJ, USA). Miglyol 812N was purchased from Fisher Scientific (NJ, USA). Cremophor® EL was purchased from Sigma-Aldrich (MO, USA). Propylene glycol (PG) was purchased from Spectrum Chemicals (CA, USA). Super Refined® Olive Oil NF-LQ (MH) was kindly provided by CRODA, Inc. (NJ, USA). Dulbecco's modified eagle medium and HyClone fetal clone III serum (fetal bovine serum [FBS]) were purchased from Fisher Scientific. DiR dye was from Life Technologies (OR, USA). Perfluoro(polyethylene glycol dimethyl ether) or PFPE oxide ( $\text{CF}_3\text{O}[\text{CF}_2\text{CF}_2\text{O}]_n\text{CF}_3$ , where  $n = 8-13$ ) was obtained from Exflur Research Corporation (TX, USA).

### Nanoemulsion preparation

Nanoemulsions were prepared using premade aqueous solution of two nonionic surfactants, Pluronic P105 (P105) and Cremophor EL as published previously with modifications [35]. Resveratrol in PG solution was prepared by dissolving 42.6 mg resveratrol in 2 ml PG stirring at 350 rpm overnight at room temperature, protected from light. PFPE oxide and/or olive oil

were added to the PG solution and vortexed at high speed for 30 s. DiR dye was added and the mixture vortexed at high speed for 30 s. Surfactant solution (11.5 ml) was added to this mixture and vortexed at high speed for 30 s. Finally, 11.5 ml deionized (DI) water was added to the mixture and vortexed at high speed for 30 s. The mixture was transferred to a pre-cooled microfluidizer (M110S, Microfluidics, MA, USA) and processed under recirculation mode for 30 pulses at inlet air pressure of approximately 80 psi and operating liquid pressure of approximately 17,500 psi. Nanoemulsion was sterilized using a sterile 0.22  $\mu\text{m}$  cellulose filter (Millex® – GS, 33 mm). Filtered nanoemulsion samples (1.5 ml) were stored at 4, 25 and 37°C. The remaining bulk of the nanoemulsion was stored at 4°C.

### Resveratrol solubility determination

Resveratrol solubility in several oils was determined with UV/VIS spectroscopy. Fifty milligrams of resveratrol powder were added to 1.0 ml of the compound to be tested. Samples were shaken for 14 h at ambient temperature, and then left, unshaken, for 4 h at ambient temperature. Samples were centrifuged at 1620 g for 10 min, and the supernatant was filtered through a 0.45  $\mu\text{m}$  filter. Filtered supernatant was diluted in methanol and quantified with UV/VIS at 320 nm.

### Nanoemulsion characterization

Nanoemulsion size distribution and  $\zeta$ -potential were measured with dynamic light scattering (DLS) using Zetasizer Nano (Malvern Instruments, Worcestershire, UK), as reported earlier [21].

### Resveratrol nanoemulsion loading

UV-VIS spectroscopy was used to assess resveratrol drug loading in all nanoemulsions. Measurements were taken at 320 nm. Samples were compared with a standard of resveratrol in methanol (concentration 3.5  $\mu\text{g}/\text{ml}$ ). Methanol was used as a blank for background correction. To prepare quantification samples for PFPE-free nanoemulsions, nanoemulsion was diluted in methanol to a total volume of 10 ml. Dilutions were performed assuming the nanoemulsion was 100% drug loading, and the target concentration for each dilution was 3.5  $\mu\text{g}/\text{ml}$ . PFPEs are not soluble in methanol, so a different approach was necessary for the accurate quantification of PFPE nanoemulsions. First, 0.5 ml nanoemulsion was diluted in methanol to a total volume of 10 ml. This first dilution was centrifuged at  $218 \times g$  for 5 min. PFPE has higher density than the other nanoemulsion components and methanol, so centrifugation caused PFPE to form a pellet. After centrifugation, the supernatant was removed

from the first dilution and diluted a second time to a total volume of 5 ml and subjected to UV-VIS measurements.

#### Resveratrol release *in vitro*

Resveratrol release from nanoemulsions was determined with Franz diffusion cells (PermeGear, Inc., Hellertown, PA 18055 USA). The receiver contained a volume of 5 ml release media. One percent weight/volume Tween 80 in phosphate buffered saline (PBS), at pH 7.4 (PBS-Tw 7.4) was used as release media. The donor compartment of two of the cells contained 350  $\mu$ l nanoemulsion and 150  $\mu$ l release media. The donor compartment of the third cell contained 350  $\mu$ l control solution and 150  $\mu$ l release media. To prepare the control solution, 21.3 mg resveratrol were dissolved in 1 ml PG by overnight stirring at 350 rpm. Resveratrol in PG solution was then diluted in release media to a total volume of 5 ml such that the concentration of this control solution was equal to the concentration of the resveratrol nanoemulsion being tested in the other two cells. The receiver and donor compartment were separated by a 30 kD regenerated cellulose membrane. The donor and receiver of each cell contained a magnetic stir bar, and cells were kept at a temperature of 37°C. A total of 22 measurements were taken over a period of 285 h. To take measurements, 300  $\mu$ l of solution were removed from the main compartment and replaced with 300  $\mu$ l fresh release media warmed to 37°C.

UV-VIS spectroscopy was used to assess resveratrol release at each time point. The method was adapted from an earlier reported study by Sessa *et al.* [9]. Briefly, calculations were performed from measurements obtained at 320 nm. Samples were prepared for UV/Vis analysis by diluting 280  $\mu$ l sample in methanol to a total volume of 3.5 ml. Samples were quantified by comparison to a standard of resveratrol in methanol (concentration 3.5  $\mu$ g/ml). Methanol was used as a blank solution and background correction for all time point measurements. Cumulative resveratrol release was calculated using Equation 1 [9].

$$Q = \frac{C_n V_t + \sum_{i=1}^{n-1} C_i V_s}{C_o V_b} * 100$$

Q was the cumulative amount of resveratrol released (%),  $C_n$  was the concentration of resveratrol ( $\mu$ g/ml) determined at the nth sampling interval,  $V_t$  was the total volume of the main compartment of the Franz diffusion cell (5 ml),  $C_i$  was the concentration of resveratrol ( $\mu$ g/ml) determined at the n-1th sampling interval,  $V_s$  was the volume of the sample removed from the main compartment at each time point (0.3 ml),  $C_o$

was the concentration of resveratrol nanoemulsion or control solution added to the donor compartment at the 0 h time point ( $\mu$ g/ml) and  $V_b$  was the volume of nanoemulsion or control solution added to the donor compartment at the 0 h time point (0.35 ml).

#### Fluorescent imaging in cells

Macrophages were cultured for 24 h in Lab Tek II Chamber Slide systems. Cultured macrophages were exposed to nanoemulsion B (10  $\mu$ l nanoemulsion/ml medium; 1 ml total) for 24 h. After removing 1 ml medium, cells were fixed in 0.3 ml of 4% paraformaldehyde for 30 min. The medium was carefully removed and washed with PBS (supplemented with 2% FBS). A stock solution of 1  $\mu$ g/ml of Hoechst dye was prepared in 2% FBS-in PBS and cells were incubated with about 0.5 ml of this stock for 10 min at room temperature. The dye solution was removed and cells were washed with 2% FBS in PBS 2 $\times$ . Images were captured on Olympus FV1200 FluoView Laser Scanning confocal microscope (Olympus America Inc, Center Valley, PA, USA). The microscope is equipped with a 20 $\times$  objective that can zoom in to 150 $\times$  magnification.

#### Flow cytometry

RAW 264.7 macrophages were cultured for 48 h in 6-well plates. After 48 h, media was aspirated and macrophages were exposed to treatment (DiD labeled, drug-free nanoemulsion) or no treatment. DiR (800 nm) is not detectable by most flow cytometry instruments. Hence, we have replaced it for the purpose of this experiment with DiD. DiD dye is chemically similar to DiR and can be used in these formulations interchangeably with DiR as reported previously [35]. Macrophages exposed to treatment received 2 ml fresh media at nanoemulsion concentration of 19.58  $\mu$ l/ml. Untreated macrophages received 2 ml fresh media. After 24 h of treatment with drug-free nanoemulsion or no treatment, media was aspirated and replaced with 2 ml fresh media containing lipopolysaccharide (LPS) at a concentration of 500 ng/ml. Cells were stimulated with LPS for 3 h. After 3 h LPS stimulation, media was aspirated and macrophages were transferred from six-well plates to 15 ml centrifuge tubes. Macrophages were centrifuged at 218  $\times$  g, room temperature for 5 min. The supernatant was aspirated and the pellet resuspended with 250  $\mu$ l fresh 2% v/v FBS in dulbecco's phosphate-buffered saline (DPBS) and 250  $\mu$ l 4% paraformaldehyde (PFA). Samples were transferred to round bottom 10 ml tubes, covered with aluminum foil, and stored at 4°C. Flow cytometry analysis was performed and data collected on BD LSR Fortessa instrument (Becton Dickinson and Company, San Jose, CA, USA).

Compound tested	Resveratrol solubility (mg/ml)
Olive oil	0.208 ± 0.004
Miglyol	0.803 ± 0.005
Propylene glycol	46.37 ± 0.232

### Macrophage viability

Macrophage viability upon exposure to increasing concentrations of nanoemulsion was assessed using Cell TiterGlo<sup>®</sup> luminescence assay as reported earlier [21].

### NO assay in macrophages

RAW264.7 cells were plated in 96-well plates at 10,000 cells/well for 24 h. Cells were exposed to resveratrol-loaded nanoemulsion (prediluted in medium) ranging from a concentration of 0–40 µl/ml of nanoemulsion (corresponding to resveratrol concentration of 0–22.4 µg/ml), free resveratrol dissolved in DMSO (2.19–35 µl/ml), resveratrol-free nanoemulsion and DMSO alone for 24 h. Fresh medium was added to unexposed cells. After 24 h, all wells were washed (2×) with medium and PBS. Bacterial toxin LPS at 200 ng/ml, diluted in medium, was added to each well with cells (exposed and unexposed) and incubated. Unexposed cells treated with LPS were designated as control, and unexposed cells without LPS activation were designated as untreated. After 24 h, supernatant was collected and NO levels were measured using a commercially available Griess reagent kit (Promega, WI, USA) according to manufacturer's instructions.

### Statistical analyzes

All analyses were performed against controls. Drug-free nanoemulsions were compared with drug-loaded nanoemulsions and to free drug in drug release studies, cell culture assays and for all colloidal stability tests. Collected data were processed and figures constructed on GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA). Where applicable we used *t*-test for comparisons between groups and one-way

(ANOVA) analysis of variance. The differences in values between groups were deemed nonsignificant for *p* > 0.05.

## Results & discussion

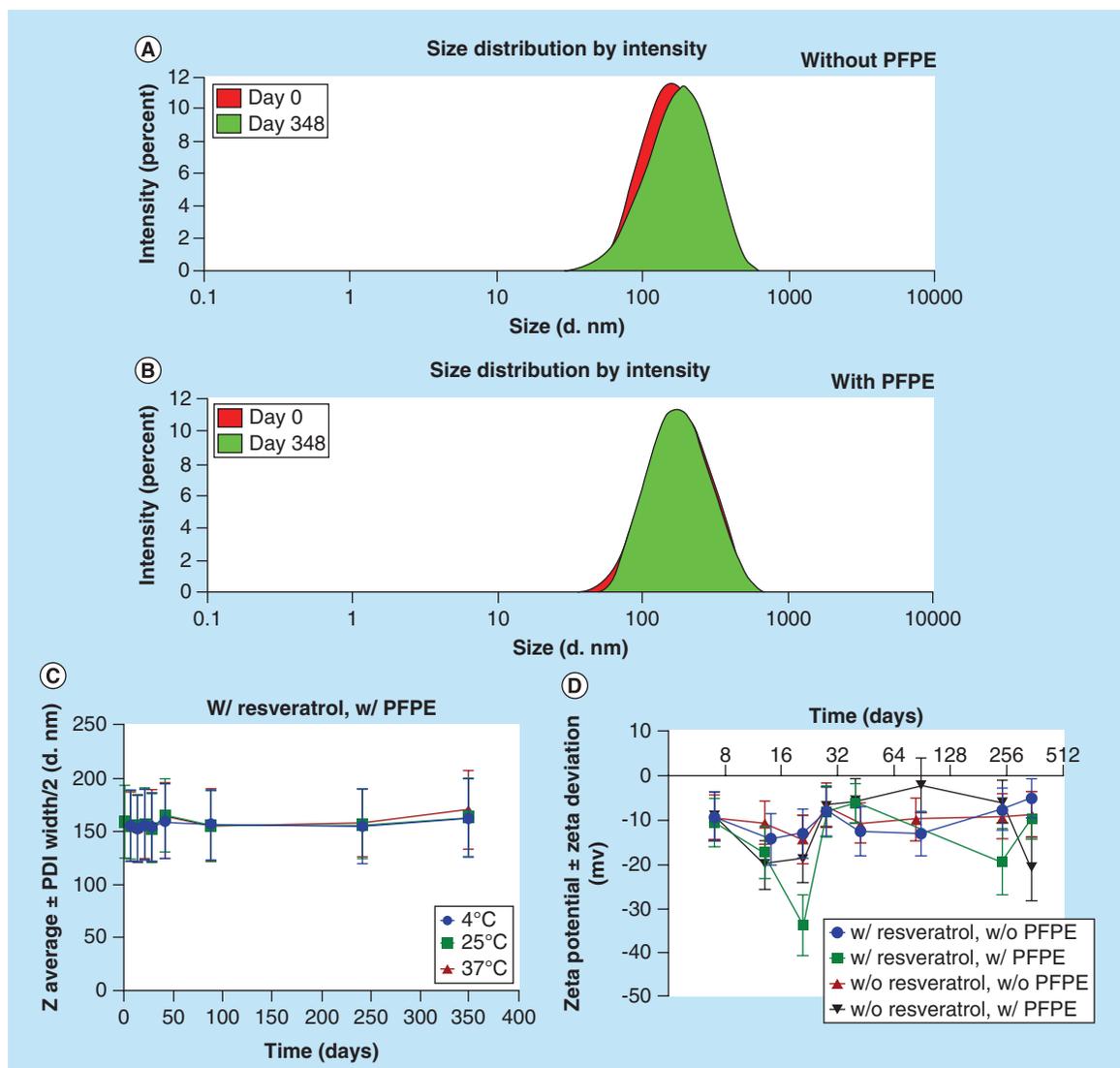
### Resveratrol nanoemulsion design

The theranostic resveratrol nanoemulsions presented here are new formulations based on our earlier reported complex triphasic systems [23,35–36]. Triphasic nanoemulsions contain a PFPE core that can be imaged with <sup>19</sup>F MRI. This PFPE core is surrounded by an organic phase that is capable of carrying poorly water soluble drugs and NIR dyes. The organic phase is stabilized with a nonionic surfactant mixture of Pluronic P105 and Cremophor EL. Previous work has shown that celecoxib-loaded nanoemulsions could be designed to target macrophages, immune cells that are involved in inflammation and pain [22–23,35–36]. Resveratrol is known to inhibit TNF-α-induced activation of nuclear factor-κB (NF-κB), a transcription factor responsible for the regulation of inflammatory signaling pathways in macrophages [37]. Additionally, NF-κB has been found to mediate tumor growth and progression [38]. We therefore hypothesized that resveratrol would be beneficial to incorporate into a macrophage-targeting nanoemulsion because unlike celecoxib, resveratrol possesses both anti-inflammatory and anticancer properties. Therefore, resveratrol nanoemulsions have potential as a versatile treatment strategy. Additionally, resveratrol is a natural compound that should not cause serious side effects.

Previously developed celecoxib nanoemulsion formulations contained Miglyol 812N oil as the internal phase and therefore were not compatible with resveratrol, as resveratrol was found to be poorly soluble in

Nanoemulsion	A	B	C	D	E	F
Resveratrol in propylene glycol	0.8	0.8	–	–	0.8	0.8
Olive oil	1.2	0.2	1.2	0.2	–	1.2
PFPE oxide	–	1.0	–	1.0	1.0	–
Micelle solution (P105/CrEL)	11.5	11.5	11.5	11.5	11.5	11.5
DiR dye (1.97 mm)	0.127	0.127	0.127	0.127	–	–
Deionized water	11.5	11.5	11.5	11.5	11.5	11.5

CrEL: Cremophor EL; DiR: 1,1'-dioctadecyl-3,3',3'-tetramethylindotricarbocyanine iodide; PFPE: Perfluoropolyether.



**Figure 1.** Effects of PFPE on size and stability of resveratrol loaded nanoemulsions. (A) Size distribution by intensity of nanoemulsion C (w/o resveratrol, w/o PFPE) at 0 and 348 days. (B) Size distribution by intensity of nanoemulsion D (w/o resveratrol, w/PFPE) at 0 and 348 days. (C) Size distribution of nanoemulsion B (with resveratrol, with PFPE) over a period of 348 days. Nanoemulsions were stored at 4, 25 and 37°C. The half width of the polydispersity index (width/two) was used as the standard deviation for size distribution measurements; (D)  $\zeta$ -potential distribution of nanoemulsions (A–D) over a period of 348 days at a storage temperature of 4°C. PDI: Polydispersity index; PFPE: Perfluoropolyether; W/: With; W/o: Without.

Miglyol 812N (Table 1). Therefore, it was necessary to develop a new nanoemulsion formulation for resveratrol. Previous studies have shown that resveratrol demonstrates good solubility in PG [11,39]. In the presented work, resveratrol solubility in PG was  $46.37 \pm 0.232$  mg/ml (Table 1). This level of solubility was more than sufficient to develop nanoemulsions that were concentrated enough to be easily administered in doses as high as 20  $\mu$ m. As doses exceeding 20  $\mu$ m are generally not observed, PG was deemed a sufficient solubilizer and was incorporated into all resveratrol nanoemulsion formulations.

In addition to PG, developed nanoemulsions contain olive oil and PFPE in the internal phase. Olive oil was chosen because it has been shown to stabilize and protect nanoemulsions against Ostwald ripening, a phenomenon characterized by the gradual increase in nanoemulsion size over time as droplets aggregate [40]. However, resveratrol is poorly soluble in olive oil (Table 1). Thus, developed nanoemulsions contained both olive oil and PG; the latter was used to solubilize the drug (resveratrol), and the former was used to stabilize the nanoemulsion. Nanoemulsion formulation compositions are summarized in Table 2. Formulations

include nanoemulsions prepared with and without PFPE, and with and without resveratrol.

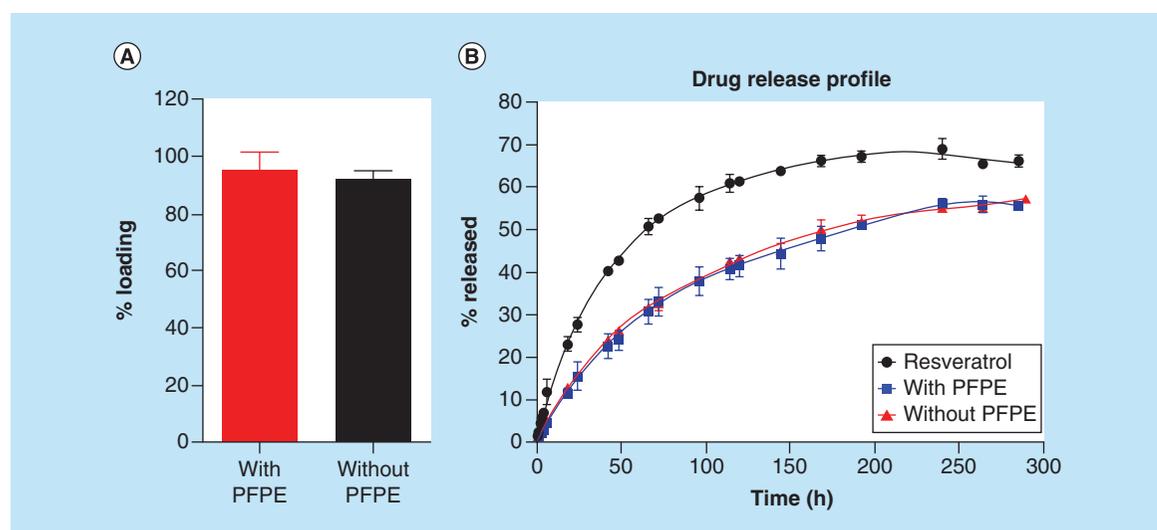
### Nanoemulsion characterization & physical stability

Nanoemulsion size,  $\zeta$ -potential and physical stability are shown in Figure 1. All prepared nanoemulsions (Table 2) show excellent colloidal stability upon storage as measured by DLS over time. No significant change in droplet diameter and polydispersity was observed for 348 days (Figure 1A–C & Supplementary Figure 1). When nanoemulsions were stored at temperatures of 4, 25 and 37°C, storage temperature did not have an effect on nanoemulsion size distribution. Further, the presence of resveratrol and PFPE did not have a significant effect on the size distribution profile (Supplementary Figure 2A) or shelf life (Figure 1A–C & Supplementary Figure 1).

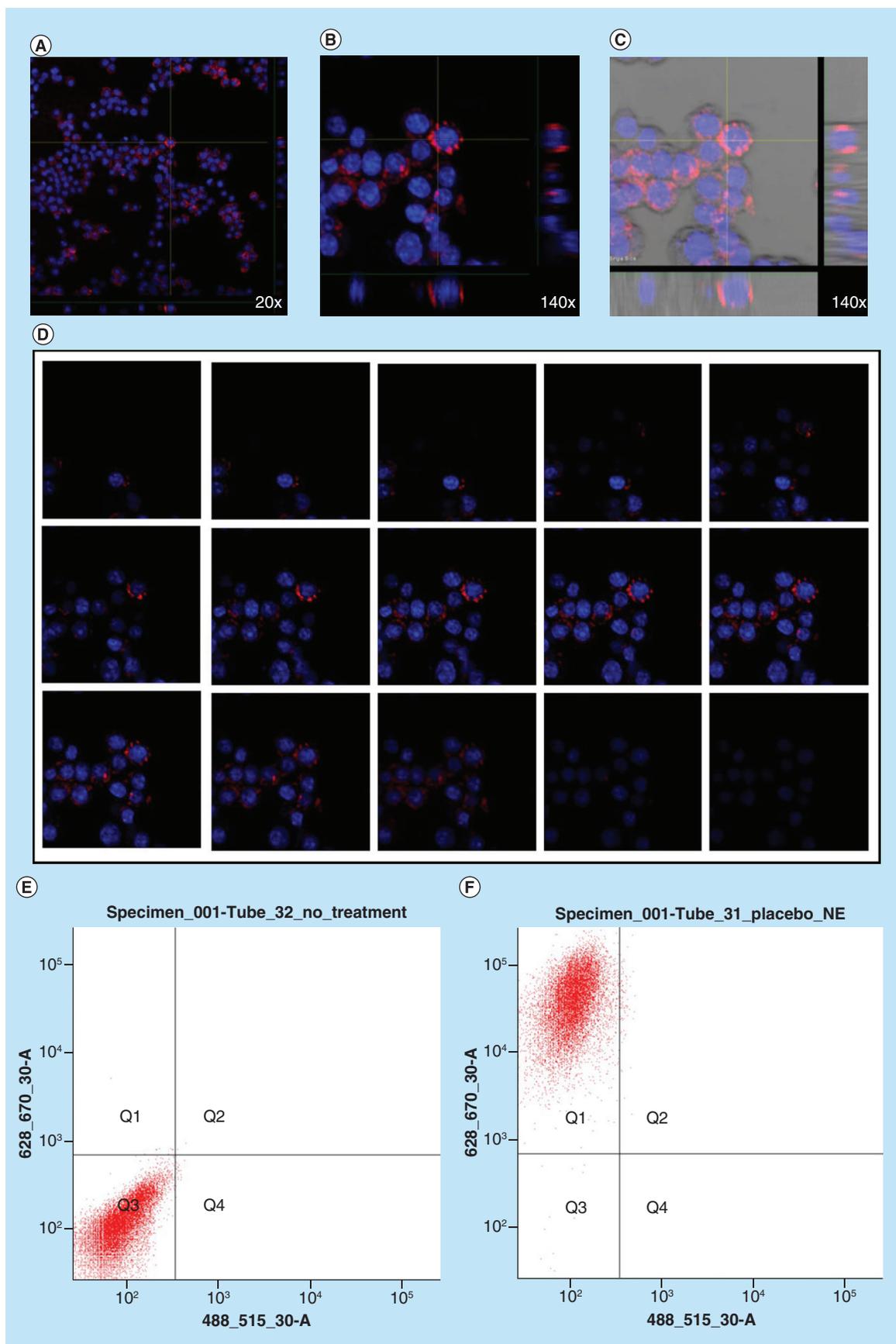
Nanoemulsions are typically stored at a low temperature to protect resveratrol from light and temperature-induced changes. When stored 4°C, average nanoemulsion diameter was  $155.1 \pm 33.1$  nm for PFPE nanoemulsions and  $140.3 \pm 30.5$  nm for PFPE-free nanoemulsions. Average nanoemulsion diameter was  $146.5 \pm 31.2$  nm for resveratrol-loaded nanoemulsions and  $149.0 \pm 32.4$  nm for resveratrol-free nanoemulsions. Small nanoemulsion droplet size differences were observed between PFPE and PFPE-free nanoemulsions (no more than 15 nm on average). Average

$\zeta$ -potential was  $-13.4 \pm 5.5$  mV for PFPE nanoemulsions and  $-10.4 \pm 5.1$  mV for PFPE-free nanoemulsions (Supplementary Figure 2B) and changes in  $\zeta$ -potential were not statistically significant for all nanoemulsions during repeated measurements for 348 days (Figure 1B). Average  $\zeta$ -potential was  $-11.0 \pm 5.2$  mV for resveratrol-loaded nanoemulsions and  $-12.8 \pm 5.4$  mV for resveratrol-free nanoemulsions (Supplementary Figure 2B). The  $\zeta$ -potential is consistent with our earlier reported nanoemulsions, which demonstrated high levels of macrophage targeting *in vivo* and *in vitro* [22–23,35–36].

Pharmacological and imaging evaluations can be confounded by colloidal destabilization under testing conditions. Therefore, the reported nanoemulsion colloidal stability was also tested under three cell culture-relevant conditions: serum-free medium, 10 and 20% serum containing medium, all at 37°C. Nanoemulsions were tested against DI water as a control, and all measurements were performed using DLS [21]. These evaluations help determine nanoemulsion stability under *in vitro* biological testing conditions, and serve as additional quality control prior to *in vivo* experiments. Nanoemulsion dilution in FBS caused a small initial increase in diameter (~10–20 nm) that stayed unchanged for 72 h when compared with nanoemulsion diluted in DI water (Supplementary Figure 3A–D). This is consistent with our earlier reports and is likely due to adsorption of proteins to the nanoemulsion sur-



**Figure 2. Resveratrol nanoemulsion drug content and drug release for nanoemulsions with and without perfluoropolyether.** (A) Nanoemulsion drug content (percent loading) determined by UV/VIS analyses for nanoemulsions with PFPE (red bar) and without PFPE (black bar). Data represent the average  $\pm$  standard deviation for individual batches of nanoemulsions produced. The presence of PFPE did not impact drug loading ( $p > 0.05$ ,  $t$ -test); (B) drug release profile at pH 7.4 for nanoemulsions with (blue) and without (red) PFPE compared with a control solution of free resveratrol (black). Nanoemulsions were assayed for drug release over 285 h in three independent runs. Drug concentration in release medium was determined by UV/VIS. Data were analyzed and curve fit produced on GraphPad Prism. There was no significant difference in drug release profile between PFPE-loaded and PFPE-free nanoemulsions as analyzed by one-way ANOVA ( $p = 0.99$ ). PFPE: Perfluoropolyether.



**Figure 3. Fluorescence imaging and flow cytometry analyses of macrophages after 24 h of exposure to fluorescently labeled resveratrol-loaded nanoemulsions (see facing page).** (A) 20× magnification of macrophages loaded with nanoemulsion (red); (B) composite z-stack fluorescent image at 140× magnification showing nanoemulsions (red) surrounding the nucleus (blue); (C) composite z-stack fluorescent and DIC (differential interference contrast) image at 140× magnification; (D) images of each individual slice from the z-stack used to obtain the composite fluorescent image shown in (B); (E) untreated cells (lower left quadrant, Q2) and (F) DiD-labeled nanoemulsion labeled cells (upper left quadrant, Q1). For flow cytometry, nanoemulsions were labeled with DiD dye and cells were exposed for 24 h before analysis. The figure shows >99% labeling of cells with nanoemulsions. Confocal microscopy was obtained on Olympus FV1200, and flow cytometry was performed on a Becton Dickinson LSR Fortessa instrument.

face, as proteins are known to form protein–surfactant complexes [41,42]. We concluded that the nanoemulsions maintain colloidal stability under *in vitro* cell culture conditions and the nanoemulsions were cleared for further testing in cells.

Nanoemulsion colloidal stability was also evaluated under drug release conditions as a precursor to Franz diffusion drug release studies. The purpose of this study was to verify that drug release media (1% Tween 80 in PBS at pH 7.4) did not affect nanoemulsion size distribution, hence suggesting colloidal stability. Nanoemulsions both with and without PFPE maintained colloidal stability in DI water and drug release media for a period of 51 h at 37°C. Centrifugation did not affect nanoemulsion stability (Supplementary Figure 3E & F).

### Resveratrol loading & release studies

Drug loading and *in vitro* drug release profiles for resveratrol nanoemulsions with and without PFPE are shown in Figure 2. To evaluate batch to batch variability, each of the nanoemulsions were produced at least twice by two independent researchers, and drug loading was assessed at time of production using UV/VIS. Resveratrol UV/VIS standard curves used in drug content determinations are shown in supplementary information (Supplementary Figure 4). The nanoemulsion without PFPE (nanoemulsion A) had average resveratrol loading of  $92.3 \pm 2.7\%$  and the nanoemulsion with PFPE (nanoemulsion C) had average resveratrol loading of  $95.4 \pm 6.4\%$  (Figure 2A). There was no significant difference in resveratrol loading between PFPE-free and PFPE-containing nanoemulsions as determined by unpaired *t*-test ( $p > 0.05$ ;  $p = 0.6$ ). To evaluate resveratrol chemical stability, we monitored resveratrol content over time using UV-VIS. Nanoemulsions were stored at 4, 25 and 37°C. We found that resveratrol loading in nanoemulsions remained stable over a period of 6 weeks under all storage conditions, and that storage temperature did not have an effect on nanoemulsion resveratrol loading (Supplementary Figure 5). These data suggest that nanoemulsions (both with and without PFPE) effectively encapsulate resveratrol and protect resveratrol against degradation. We concluded that PFPE does not have an effect on nanoemulsion

resveratrol loading and that PFPE has no effect on nanoemulsion drug content retention over time.

*In vitro* release from resveratrol nanoemulsions with and without PFPE was evaluated using Franz diffusion analysis. A solution of free resveratrol in PG diluted in release media to a concentration matching that of the resveratrol-loaded nanoemulsion was used as a control. The release studies were performed under sink conditions to ensure that resveratrol solubility did not interfere with its release. Nanoemulsions and control solution were evaluated for a period of 285 h. A maximum resveratrol release of approximately 57% was observed for both nanoemulsions E (with PFPE) and F (without PFPE), and a maximum resveratrol release of approximately 70% was observed for the free resveratrol control (Figure 2B, black curve). Resveratrol nanoemulsion release profiles are consistent with Hung *et al.*, who reported up to 55% resveratrol release from a nanoemulsion containing coconut oil and vitamin E [43]. These data suggest that the resveratrol release from the nanoemulsion is not affected by the presence of PFPE (verified using one-way ANOVA,  $p > 0.05$ ).

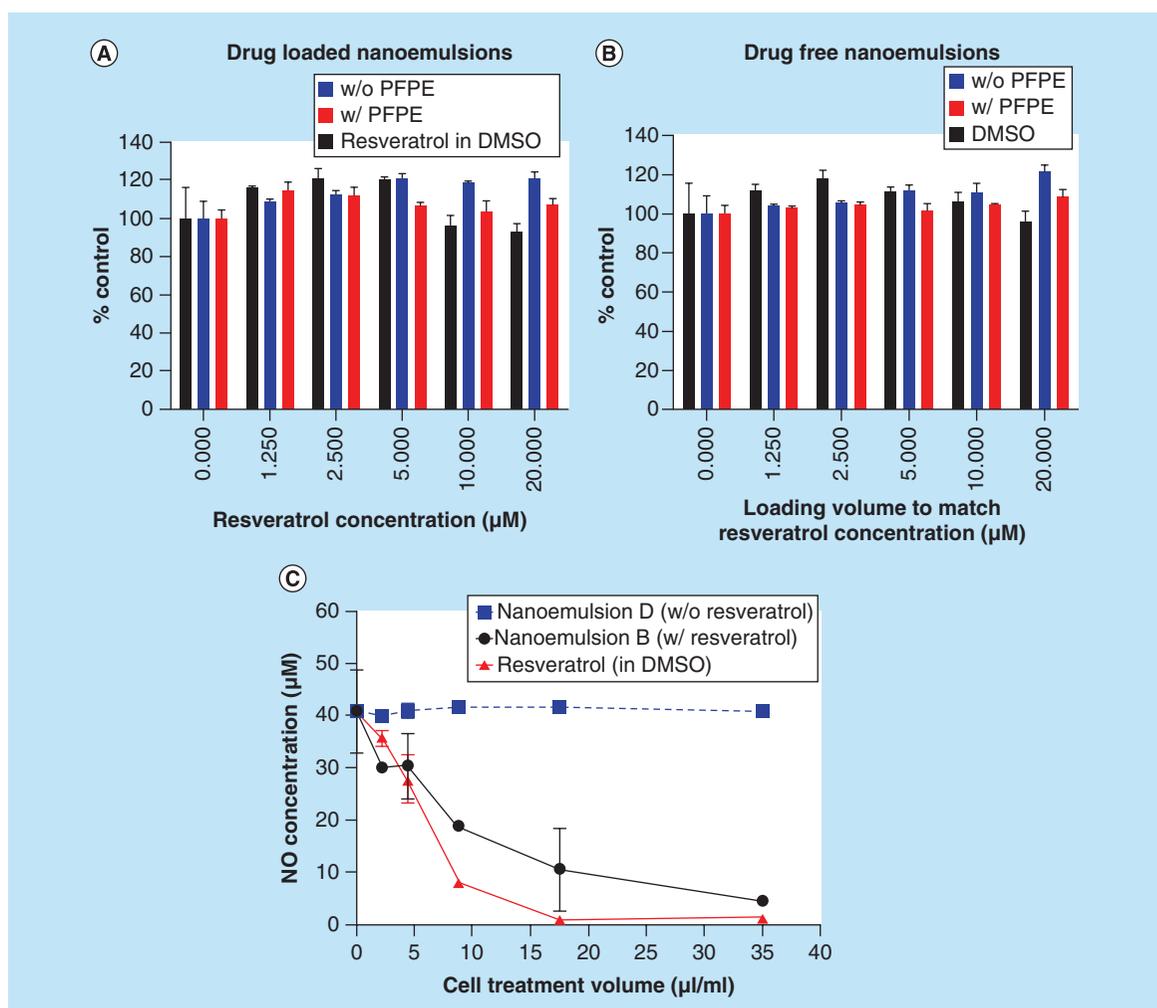
### Macrophage uptake & imaging studies

Resveratrol has been shown to decrease the production of proinflammatory chemokines and cytokines through inhibition of NF-κB in macrophages [44]. We therefore hypothesized that targeted delivery of resveratrol directly to the macrophage would improve its anti-inflammatory efficacy. To investigate the effects of encapsulated resveratrol in cells, we used an established macrophage cell line, RAW 264.7 cells. Resveratrol-loaded nanoemulsions presented here were successfully internalized by macrophages, as imaged with confocal fluorescence microscopy (Figure 3A–D). Fluorescently labeled nanoemulsions are visible within the macrophage in the *xy*-, *yz*- and *xz*-planes (Figure 3C). Further, nanoemulsions are visible in each individual slice of a z-stack (Figure 3D). These results suggest that nanoemulsions are fully internalized by the macrophages, and that nanoemulsions are not associated with the surface of the macrophage. These images fully correlate to earlier studies where live imaging was performed for PFPE nanoemulsions [31,36]. In previous studies, nanoemul-

sions were evaluated using confocal imaging on both live and fixed cells, and no difference in nanoemulsion uptake by macrophages was observed for either imaging approach [23,45]. The fixation of cells for the purpose of imaging, if done correctly, retains membrane integrity, and here, we have demonstrated that nanoemulsions reside in the cytoplasm of the macrophage. Macrophage internalization of nanoemulsions was also confirmed with flow cytometry, which showed >99% macrophage labeling with nanoemulsion (Figure 3E & F). Resveratrol nanoemulsion uptake in macrophages was determined to be concentration dependent (Supplementary Figure 6).

### Macrophage viability & NO production

Figure 4A & B shows the effect of developed nanoemulsion formulations on RAW 264.7 macrophage viability. For drug-loaded nanoemulsions, free resveratrol in DMSO was used as a free drug control, and for drug-free nanoemulsions, DMSO alone was used as a negative (vehicle) control. Macrophage viability was <10% when treated with doxorubicin, a toxic agent used as a positive control, at a dose of 5  $\mu\text{m}$  in these assays (Supplementary Figure 7) [46]. All nanoemulsions reported here showed no impact on cell viability in LPS-stimulated macrophages at doses up to 20  $\mu\text{m}$  (Figure 4A & B). These findings are impor-



**Figure 4. Nanoemulsions *in vitro* evaluations in macrophages.** (A & B) Cell viability measured by luminescence-based assay in macrophages (RAW 264.7). Cells were exposed to varied concentrations of drug (0–20  $\mu\text{M}$ ) in resveratrol-loaded nanoemulsion (with or without PFPE) and DMSO (free drug control). Cell viability was performed with 24 h LPS (500 ng/ml) stimulation. (A) Comparison of resveratrol-loaded nanoemulsions to free drug; (B) comparison of resveratrol-free nanoemulsions to DMSO; (C); Production of NO in macrophages assessed after LPS treatment. After LPS treatment, cells were exposed to resveratrol-free nanoemulsion (nanoemulsion D, with PFPE), resveratrol-loaded nanoemulsion (nanoemulsion B, with PFPE), or free drug (resveratrol) in DMSO. Macrophages exposed to resveratrol-loaded nanoemulsion showed a decrease in NO production that is comparable to that of macrophages exposed to free resveratrol in DMSO. LPS: Lipopolysaccharide; PFPE: Perfluoropolyether; W/: With; W/o: Without.

tant for two reasons. First, NO measurements require LPS stimulation, so it is critical that resveratrol-loaded nanoemulsions do not affect macrophage viability of LPS-stimulated macrophages at the highest dose of resveratrol nanoemulsion. Any macrophage death caused by resveratrol-loaded nanoemulsions would confound the results of a NO study. Second, in future live animal studies we will aim to deliver resveratrol to activated proinflammatory macrophages. Therefore, it is important to deliver high concentrations of resveratrol-loaded nanoemulsion to activated macrophages without causing macrophage loss of function and/or death, as these effects could confound our pharmacological findings *in vitro* and *in vivo*.

Proinflammatory, M1, macrophages produce several mediators involved in oxidative processes, such as NO [20]. These mediators help to eliminate foreign microorganisms at the site of inflammation [20]. However, if levels of M1 macrophage infiltration become too high at the site of inflammation, increased levels of NO can cause damage to healthy tissue and lead to increased, chronic inflammation [20]. Previously, celecoxib-loaded nanoemulsions were shown to suppress prostaglandin release from LPS-activated macrophages [18,35]. Here, macrophages were exposed to resveratrol-free nanoemulsions, resveratrol-loaded nanoemulsions or free resveratrol in DMSO at a concentration of 20  $\mu\text{m}$ . Macrophages were then stimulated with LPS to induce NO production. Macrophage exposure to resveratrol-free nanoemulsion had no effect on levels of NO production (Figure 4C). Macrophages exposed to resveratrol-loaded nanoemulsion showed a decrease in NO production that is comparable to that of macrophages exposed to free resveratrol in DMSO. Figure 4A & B shows that at resveratrol concentrations of 20  $\mu\text{m}$ , neither resveratrol-loaded nanoemulsion nor free resveratrol in DMSO had a significant impact on macrophage viability. Therefore, the decrease in NO shown here (Figure 4C) is due to the effect of resveratrol on production of NO and not by macrophage decrease in metabolic activity or cell death.

## Conclusion

We report here, for the first time, a resveratrol-loaded theranostic nanoemulsion carrying two imaging agents, PFPE (MRI agent) and DiR (optical imaging agent). Data presented suggest that theranostic nanoemulsion formulations successfully encapsulate resveratrol, maintain high drug loading over time (>90%) and show remarkable colloidal stability (348-day shelf life) and extended release profile (285 h). The presence of PFPE did not have an effect on nanoemulsion size,  $\zeta$ -potential, physical stability, drug loading, *in vitro* release profile or behavior in macrophages. Nanoemul-

sions were nontoxic at doses of up to 20  $\mu\text{m}$  and were readily internalized by macrophages *in vitro*, and resveratrol-loaded nanoemulsions significantly decreased the production of NO in LPS-activated macrophages *in vitro* without causing cell viability changes. The presented formulation strategy opens a new avenue for theranostics development and introduces a new approach for imaging-supported delivery of resveratrol.

## Future perspective

Nanoemulsions show great potential to provide safe, effective delivery of lipophilic natural compounds (such as resveratrol) known to suffer poor bioavailability or chemical instability. In a recent editorial, McClements included nanoemulsions as nutraceutical delivery systems for producing foods with the purpose of disease prevention [47]. We agree and argue that to fully understand the benefits of nanoemulsions as nutraceutical carriers and food enhancing formulations, it is crucial to ascertain information regarding their *in vivo* biodistribution and efficacy in reaching select tissues and organs. The incorporation of imaging agents into drug or natural product delivering nanoemulsions provides access to this crucial information and can help us advance nanoemulsions into clinical applications of a broader scope. The presented work demonstrates that the  $^{19}\text{F}$  MRI agent PFPE can be incorporated into resveratrol nanoemulsions without affecting nanoemulsion properties. Therefore, in future studies, it should be possible to use PFPE nanoemulsions and their PFPE-free counterparts interchangeably, depending upon imaging needs at the time. The metabolic and biologic inertness of PFPE would enable monitoring of PFPE nanoemulsion biodistribution over long periods of time. However, other nanoemulsion components are inert. The formulations reported here incorporate cremophor EL at concentrations significantly lower than those used in extensively studied formulations in humans known to cause significant toxicity [48]. Furthermore, these formulations incorporate approximately 50% less cremophor EL than our previously published nanoemulsions successfully used *in vivo* in a murine inflammation model [22]. In future translational studies we will aim to eliminate cremophor EL from nanoemulsions altogether.

This study utilized NO assay as a limited assessment of the anti-inflammatory action of nanoemulsions. Though promising results were obtained, we want to emphasize that further studies are needed to fully investigate the effectiveness of these theranostic resveratrol formulations in models of inflammatory diseases. As *in vivo* behavior of nanoemulsions may be highly impacted by oil content and composition, additional tests (e.g., transmission electron microscopy) are underway to further confirm the triphasic nature of presented nanoemulsions

under biologically relevant conditions. As triphasic colloidal systems (perfluorocarbon/hydrocarbon/water) are not commonly produced on a nanoscale level, the methodology for their successful imaging presents a unique challenge. Hence, these studies are beyond the scope of this preliminary communication report and will be presented in an upcoming publication.

#### Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: [www.future-science.com/doi/full/10.4155/tde-2016-0050](http://www.future-science.com/doi/full/10.4155/tde-2016-0050)

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#### Executive summary

##### Need for incorporation of imaging agents into resveratrol nanoemulsions

- Resveratrol's efficacy in clinical studies varies, possibly due to resveratrol's poor water solubility, bioavailability and chemical stability – nanoemulsions have been shown to improve these properties.
- Nanoemulsion biodistribution is difficult to study *in vivo*, and therefore, it is difficult to directly connect cell- or tissue-specific resveratrol nanoemulsion delivery with pharmacological outcomes.
- The incorporation of two complementary imaging modalities (1,1'-diiodo-3,3',3'-tetramethylindotricarbocyanine iodide [DiR] dye for near infrared and perfluoropolyether [PFPE] for <sup>19</sup>F MRI) has the potential to dramatically increase understanding of how nanoemulsions improve resveratrol bioavailability and *in vivo* efficacy by providing an imaging signature of their distribution to tissues and cells.

##### Effects of imaging agents (PFPE) on drug delivery properties of nanoemulsions

- The <sup>19</sup>F MRI agent PFPE is both hydrophobic and lipophobic, and therefore may interfere with resveratrol nanoemulsion drug delivery properties, such as drug loading, drug release and colloidal stability.
- Resveratrol-loaded nanoemulsions with and without PFPE are reported, and it was demonstrated that the presence of PFPE did not impact nanoemulsion size,  $\zeta$ -potential, colloidal stability, drug loading or drug release.

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