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PII: S0022-3549(24)00018-2
DOI: https://doi.org/10.1016/j.xphs.2024.01.013
Reference: XPHS 3311

To appear in: *Journal of Pharmaceutical Sciences*

Received date: 12 October 2023
Revised date: 22 January 2024
Accepted date: 22 January 2024

Please cite this article as: Cinzia Cimino , Angela Bonaccorso , Barbara Tomasello , Giovanni Anfuso Alberghina , Teresa Musumeci , Carmelo Puglia , Rosario Pignatello , Agostino Marrazzo , Claudia Carbone , W/O/W microemulsions for nasal delivery of hydrophilic compounds: a preliminary study, *Journal of Pharmaceutical Sciences* (2024), doi: https://doi.org/10.1016/j.xphs.2024.01.013

Highlights

- The preformulative screening allowed the development of W/O/W microemulsions adequate for the intended nasal administration.
- Accelerated stability studies demonstrated a long-term stability for the prepared W/O/W microemulsions.
- W/O/W microemulsions showed good mucoadhesive properties, which could guarantee the adhesion on the nasal mucosa.
- A rapid and sustained release of fluorescein from the neutral W/O/W microemulsion was obtained.
- Neutral W/O/W microemulsion represents a promising system due to its high biocompatibility on HFF1 and Calu-3 cell lines.

Research Article

W/O/W microemulsions for nasal delivery of hydrophilic compounds: a preliminary study

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Abstract:
The administration of hydrophilic therapeutics has always been a great challenge because of their low bioavailability after administration. For this purpose, W/O/W microemulsion resulted to be a potential successful strategy for the delivery of hydrophilic compounds, interesting for the nasal mucosal therapy. Herein, an optimized biphasic W/O microemulsion was designed, through a preliminary screening, and it was inverted in a triphasic W/O/W microemulsion, intended for the nasal administration. In order to enhance the mucosal retention, surface modification of the biphasic W/O microemulsion was performed adding didodecyldimethylammonium bromide, and then converting the system into a cationic triphasic W/O/W microemulsion. The developed samples were characterized in terms of droplet size, polydispersity, zeta potential, pH and osmolality. The physical long-term stability was analyzed storing samples at accelerated conditions (40±2°C and 75±5% RH) for 6 months in a constant climate chamber, following ICH guidelines Q1A (R2). In order to verify the potential retention on the nasal mucosa, the two triphasic systems were analyzed in terms of mucoadhesive properties, measuring the *in vitro* interaction with mucin over time. Furthermore, fluorescein sodium salt was selected as a model hydrophilic drug to be encapsulated into the inner core of the two triphasic W/O/W microemulsions, and its release was analyzed compared to the free probe solution. The cytocompatibility of the two platforms was assessed on two cell lines, human fibroblasts HFF1 and Calu-3 cell lines, chosen as pre-clinical models for nasal and bronchial/tracheal airway epithelium.

Keywords: Drug delivery system; Emulsions; Nasal drug delivery; Physical stability; Preformulation; Self-emulsifying.

1. Introduction

Currently, the need to obtain increased drug efficacy or reduced side effects led scientific community to develop innovative drug delivery systems for hydrophilic molecules, which are becoming more relevant in therapy. Many therapeutical compounds, such as vaccines, nucleic acids, proteins and some anti-cancer molecules, have a hydrophilic nature with poor permeability, low intracellular absorption, rapid enzymatic degradation and clearance, moderate distribution, low therapeutic index, and poor pharmacokinetic profile[1]. To overcome these limitations, the focus of researchers is aimed to the development of Drug Delivery Systems (DDS) which are effectively capable to vehiculate hydrophilic molecules, since these compounds have weak interactions with many drug carriers, resulting in a low encapsulation efficiency, unwanted loss and initial burst release[2]. Having an inner water core, W/O emulsions could be suitable for the encapsulation of hydrophilic molecules. They present the advantage of providing a higher solubilization compared to simple

micellar dispersions, assuring greater kinetic stability. Furthermore, their small droplets size provides a pathway to drastically increase the drug dissolution rate and subsequently their systemic bioavailability[3]. Nevertheless, W/O emulsions could present critical issues because of their thermodynamic instability; they are prone to demulsification, eventually leading to bulk phase separation, sedimentation, coalescence and flocculation. Furthermore, other factors can result in physically instability for lipid oxidation, such as temperature, ionic strength, and oxygen content, which can severely restrict W/O emulsion development and application in therapeutics[4]. Otherwise, in order to enhance the delivery of drugs, multiple emulsions, such as water-oil-water (W/O/W) ones, also called double or triphasic emulsions, have been developed. They consist of a primary water in oil (W/O) emulsion of water droplets dispersed in an oily phase (primary biphasic system), which is then dispersed in an external water phase, thus leading to the formation of the W/O/W double emulsion (secondary triphasic system). Due to their composition and structure, W/O/W emulsions ensure the absorption of hydrophilic compounds and can also be exploited for simultaneously transporting hydrophilic and lipophilic compounds in a single vehicle[5]. The classification of the emulsions is based on their droplet size and thermodynamic stability. Considering droplets dimensions, emulsions could be divided into: macroemulsions (>400 nm), nanoemulsions (200-400 nm) and microemulsions (<200 nm)[6]. Nano- and micro- emulsions' size ranges actually are not strictly defined, and the two terms are frequently used ambiguously in the literature[7]. Another difference between the two emulsive platforms is the preparation method used, since nanoemulsions are produced using high-energy methods while microemulsions can be obtained by low-energy techniques[6]. Herein, the term microemulsion is used following these considerations[8], therefore referring to system with droplet size lower than 200 nm, obtained by low-energy Phase Inversion Temperature (PIT) method.

Nowadays, the scientific community is focusing its interest on the potential use of the nasal route for drug delivery, which represents an interesting strategy to deliver drugs locally, systemically, and directly to the brain (crossing over the Blood Brain Barrier, BBB) and being in direct contact with the external environment. Compared to the oral and parenteral routes, nasal administration presents different benefits, including being needle free, potentially self-administrable and non-invasive, allowing a great uptake surface area, avoiding first pass rate metabolism[9], eliminating the gastrointestinal drawbacks and side effects and guaranteeing the possibility to obtain therapeutical drug levels with a faster onset of drug activity[10]. Moreover, intranasal route is preferred in children and elderly people since it enables a better patient compliance[11]. One of the drawbacks of the nasal administration route is the low bioavailability of hydrophilic and high molecular weight (HMW) peptide and protein drugs, which is normally less than 1%[12]. However, even if nasal administration seems to be potentially efficient, the use of free drug presents several limitations, related to its chemical-physical features, such as their weak mucosal membrane permeability, but also to physiological mechanism, because of the mucociliary clearance and enzymatic degradation[12–15]. Based on these considerations, the encapsulation into DDS could be a successful strategy.

For the best of our knowledge, the microemulsive systems discussed in literature are usually O/W systems developed for the nose-to-brain delivery[16–18], therefore with a different target site to be reached with specific features. Moreover, the few systems developed for the nasal mucosal administration were O/W nanoemulsions prepared involving the use of an organic solvent, such as ethanol[19–21]. Basing on these considerations, the goal was the design of a triphasic W/O/W microemulsion for nasal mucosal delivery, prepared avoiding the use of organic solvents through the PIT method – which, to the best of our knowledge, has been only reported once for the production of a triphasic W/O/W nanoemulsion for dermal delivery[22] – and being able to encapsulate a hydrophilic drug, also with the possibility of a combined administration with a lipophilic drug.

In the present work, different raw materials were screened to select the more adequate components to obtain the primary W/O biphasic microemulsion (ME2); subsequently, the produced ME2 was inverted in order to produce an optimized secondary triphasic W/O/W microemulsion (ME3). The optimized ME2 was also modified through the addition of didodecyldimethylammonium bromide (DDAB), namely ME2+, and then inverted to obtain the cationic ME3+. The triphasic microemulsions were prepared using the PIT method, selected for being a green eco-friendly and organic solvent-free preparation method, which allows to avoid the use of organic solvents and makes it possible to avoid the employment of high-energy techniques which are not compatible with some potentially encapsulated molecule[23,24]. Droplets size (Z-ave) and polydispersity (PDI), as well as zeta potential (ZP), were assessed using Photon Correlation Spectroscopy (PCS), and pH and osmolality were measured. In order to analyze the physical long-term stability, samples were stored at accelerated conditions (40±2°C and 75±5% RH) for 6 months in a constant climate chamber, following ICH guidelines Q1A (R2)[25]. ME3 and ME3+ were also investigated to evaluate their mucoadhesive properties. Different concentrations of both formulations and different treatment times were analyzed to assess *in vitro* biocompatibility on human fibroblast (HFF1) and human airway epithelial (Calu-3) cell lines, selected chosen as pre-clinical models for nasal and bronchial/tracheal airway epithelium. Fluorescein sodium salt was encapsulated as hydrophilic model drug into the inner aqueous core of the biphasic W/O microemulsions

ME2 and ME+. The obtained fluorescein-loaded ME3 and ME3+ were characterized and dialysis bag studies were performed to evaluate the probe release.

2. Materials and Methods

2.1. Materials

Kolliphor® RH40 was provided by BASF Italia S.p.a. (Cesano Modena, Italy); Oleoyl Macrogol-6 Glycerides (Labrafil®) was a gift from Gattefossé Italia s.r.l. (Milano, Italy); Isopropyl myristate (IPM) and Triglyceride caprylic-capric (Tegosoft CT, Miglyol 812) were purchased from Farmalabor (Canosa di Puglia, Italy). Tween® 80 (Polysorbate 80), Span® 80 (Sorbitan monooleate), Didodecyldimethylammonium bromide (DDAB), Fluorescein sodium salt, Tris (hydroxymethyl)aminomethane buffer, Phosphate Buffered Saline pH 7.4 (PBS) were bought from Merck (Darmstadt, Germany). Mucin (mucin from porcine stomach type II), NaCl, $NaHCO_3$, $CaCl_2 \cdot 2H_2O$ and KCl were purchased from Merck. Regenerated cellulose membranes (Spectra/Por CE; Mol. Wet. Cutoff 3500) were supplied by Spectrum (Los Angeles, CA, USA). All materials for the biological assays were purchased from Merck. All solvents (LC grade) were from VWR International (Milan, Italy).

2.2. Preformulative studies on biphasic ME2 and triphasic ME3 and preparation method

2.2.1 Materials screening for ME2 composition

Materials to be used to produce the primary W/O biphasic microemulsion (ME2) were screened, selecting Labrafil® or Span® 80 as surfactants, Kolliphor® or Tween® 80 as co-surfactants, and Tegosoft CT or IPM as liquid lipids. To determine the optimal quantitative composition of ME2, water phase was fixed at 10% w/V[26], the following equations were used to calculate the percentages of surfactants (1) and co-surfactants (2) and the amount of oil was selected up to 100%.

$$\%_{SURFACTANT} = HLB_{TOT} - HLB_{SURFACTANT} \quad (1)$$

$$\%_{CO\text{-}SURFACTANT} = HLB_{TOT} - HLB_{CO\text{-}SURFACTANT} \quad (2)$$

The raw materials were considered in different combinations, as reported in Supplementary Table 1, obtaining 8 samples, from ME2-A to ME2-H, each one composed by: 10.0% of water, 12.8% of surfactant, 6.8% of co-surfactant and 70.4% of oil.

2.2.2 ME2 preparation method

Primary W/O biphasic microemulsions (ME2-A to ME2-H) were prepared mixing the established amounts of surfactant and co-surfactant at 300 rpm, subsequently adding the oil at 500 rpm and finally dropping the internal water phase under constant stirring at 500 rpm.

2.2.3 Photon Correlation Spectroscopy (PCS) analysis of ME2

The produced primary W/O biphasic microemulsions (ME2) were analyzed through a Zetasizer Nano S90 (Malvern Instruments, Malvern, UK), using quartz cuvettes, in order to measure the mean droplets size (Z-ave) and polydispersity (PDI) of the inner water phase.

2.2.4 ME2-A stirring time optimization

The optimization of ME2-A preparation was performed analyzing different stirring times after the dropwise addition of the internal water phase (5, 30, 60, 90, 120, 150 min). The obtained samples ME2-A_5, ME2-A_30, ME2-A_60, ME2-A_90, ME2-A_120, ME2-A_150, were analyzed using Photon Correlation Spectroscopy described above (paragraph 2.2.3).

2.2.5 ME3-A composition screening

In order to obtain the secondary triphasic W/O/W microemulsion (ME3-A), Tween® 80 was added to ME2-A at different concentrations (0%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% w/V). PIT method was used to invert the biphasic ME2-A in TRIS external phase at different dilutions (1:3, 1:6, 1:9) under constant mixing at 500 rpm for 10 min. The obtained ME3-A samples were analyzed using Photon Correlation Spectroscopy as described below (paragraph 2.3.1).

2.2.6 ME3-A surface modification

The optimized ME3-A was modified with the cationic lipid DDAB, which was added at a concentration of 0.15% w/V into the inner biphasic microemulsion (obtaining ME2+A), before its inversion to triphasic microemulsion (obtaining ME3+A).

2.2.7 Fluorescein loading
Fluorescein, as a hydrophilic model drug, was loaded into the inner water phase of ME2-A and ME2+A at a concentration of 1 mg/mL, before their inversion to triphasic microemulsions, thus obtaining F-ME3-A and F-ME3+A, respectively. Precautions were made to prevent the occurrence of fluorescein photodegradation, both during the storage and during the experiments.

2.3. Physico-chemical and technological characterization of triphasic ME3s
2.3.1. Photon Correlation Spectroscopy (PCS) analysis of ME3s
Zetasizer Nano S90 (Malvern Instruments, Malvern, UK) was also employed to analyze the secondary triphasic W/O/W microemulsion (ME3-A), using polystyrene cuvettes to measure Z-ave and PDI, and folding capillary cuvettes for measuring ZP. Three analyses of each sample were performed, and results are reported as mean ± SD.

2.3.2. Osmolality and pH measurements
Osmolality of the samples was analyzed using an osmometer (Osmomat 3000, Gonotec, Berlin, Germany) previously calibrated using ultra-purified water and physiological solution.
A pH-meter (Mettler Toledo, Milano, Italy) was used to measure samples pH, after calibration with pH 4.0, 7.0 and 10.0 solutions.

2.3.3. Accelerated stability studies
ICH guidelines Q1A (R2)[25] were followed to assess accelerated stability of blank ME3-A and ME3+A, through the incubation in constant climatic chamber (BINDER GmbH, KBF-S 115 E6, Tuttlingen, Germany) at accelerated conditions (40±2°C temperature and 75±5% RH) for 6 months. Z-ave and PDI were measured each month, for 6 months of storage.

2.4. *In vitro* studies
2.4.1. Mucoadhesion studies
An *in vitro* method based on the evaluation of two parameters (turbidimetry and ZP) was used to assess the mucoadhesive properties of the secondary triphasic W/O/W microemulsions (ME3-A and ME3+A). Briefly, mucin (0.1% w/v) was suspended in simulated nasal fluid (SNF: 2.192g NaCl, 0.145g $CaCl_2$ and 0.745g KCl in 250 mL of double distilled water; pH 5) and stirred overnight to allow its complete dispersion. The interaction between each sample and mucin was determined by mixing equal volumes of mucin dispersion and sample suspension for 15 min at 25°C. After 0.5, 1, 2 and 3h of incubation at 37°C, turbidimetry and ZP were assessed. In particular, turbidimetric measurements were evaluated comparing the absorbances at 650 nm by UV-Vis spectrophotometer (UH5300 UV-Visible Double-Beam Spectrophotometer, Hitachi Europe, Milan, Italy) of the native mucin and each dispersion.

2.4.2. Probe release
In vitro release of fluorescein from F-ME3-A and F-ME3+A, compared with fluorescein solution, was evaluated using the dialysis bag method. After 24h of moistening in PBS medium, dialysis tubes (Spectra/Por® membranes, MWCO 3.5 kDa) were filled with 1 mL of each sample and incubated in 20 mL of PBS medium, maintained at 37°C under stirring at 300 rpm. At selected time-points (from T0 to 24h), 1 mL of medium was withdrawn and replaced with 1 mL of fresh medium to guarantee pseudo-sink conditions. Each sample was analyzed by UV-VIS spectrophotometer at 490 nm in order to quantify the amount of fluorescein released during time (calibration curve from 0.6 μg/mL to 15.0 μg/mL; R^2=0.9914).

2.4.3. Cell cultures and treatments
HFF1 cells (ATCC, SCRC-1041TM) were cultured in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% v/v fetal bovine serum and (50 IU/mL) penicillin/(50 μg/mL) streptomycin. Calu-3 cells (Calu-3, HTB-55™) were grown in Minimum Essential Medium Eagle (MEM) supplemented with 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% MEM non-essential amino acid solution, 1% L-glutamine and 100 U/mL penicillin, and 100 µg/mL streptomycin (PAA Laboratories GmbH, Austria) antibiotic solution. After seeding, the cells were maintained in a controlled environment at 37°C, 5% CO_2 concentration, and 95% relative humidity[27].
For the treatment, the ME3-A and ME3+A were tested at different oil concentrations (8.81 mg/mL, 5.87 mg/mL and 3.52 mg/mL), and at different timepoints (0.5, 1, 2 and 3h).

2.4.4. Cytotoxicity evaluation

The cytotoxicity of the triphasic W/O/W microemulsions was assessed by MTT[28] in HFF1 and Calu-3 human cell lines. The cells were seeded in a 96-well plate ($1x10^4$ cells/well) and incubated for 24h to allow adherence. Both the formulations were diluted in media, filtered using 0.22 μm Millipore Express PES membrane and added to cells as described in the previous paragraph. After the treatments, the culture media was discarded and the cells were rinsed with 1X PBS, added with fresh MTT solution (500 µg/mL in culture media) and re-incubated for 2h. Then, MTT solution was replaced with DMSO for allowing the dissolution of formazan crystals. The optical density (OD) was measured at 550 nm using a microplate reader (Synergy HT multi-mode microplate reader, BioTek, Milano, Italy). Cell viability (%) was expressed as a percentage relative to the untreated control cells (negative control, cells treated with media alone), whose value was equal to 100%. Sodium dodecyl sulfate (SDS) at 0.2% w/V was used as positive cell death control.

2.5. Statistics

For the characterization of blank and loaded formulations reported in Table 2, two-way ANOVA with Bonferroni's multiple test was performed, comparing the two blanks and also each loaded sample with the respective blank. For accelerated stability studies, mucoadhesion turbidimetric assay and cytocompatibility studies, two-way ANOVA with Dunnett's multiple comparisons tests were performed. Two-way ANOVA with Tukey's multiple comparisons test was used for mucoadhesion ZP analysis. Significance was calculated applying $p<0.05$ as the minimum level. All the analysis were performed using Prism 9.5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results and discussion

3.1. Preformulative studies on biphasic ME2 and characterization

3.1.1 Materials screening for ME2 composition

Labrafil® and Tween® 80 were selected as surfactants, while Kolliphor® and Span® 80 were chosen as co-surfactants for the preliminary studies on the preparation of the biphasic W/O ME2. IPM and Tegosoft CT were investigated as the oily phase. Labrafil® is a biocompatible and biodegradable non-ionic amphiphilic PEG derivative (HLB=9), which demonstrated to be safe when used in the formulation of microemulsions for intranasal administration[29]. Span® 80 (HLB=4.3) and Tween® 80 (HLB=15) are widely used in nanoformulations for their GRAS (generally recognized as safe) status. In particular, Tween® 80 was reported to be safe on nasal tissue[30] and to promote steric stabilization of MEs[31]. Kolliphor®, a non-ionic surfactant (HLB=14-16), was demonstrated to stabilize hydrophilic compounds in emulsive systems[32]. Tegosoft CT (HLB=11) was considered in this screening because of its produce small and stable MEs[33]. IPM (HLB=11.5) was employed because of its ability to enhance nasal absorption of active molecules loaded into MEs[34], even if its concentration has to be maintained lower than 5% v/v to obtain a cell viability higher than 70%, due to its activity as penetration enhancer[35].

In order to obtain the quantitative composition of the primary W/O microemulsion ME2, firstly the water content was set at 10% w/V, basing on previous literature studies[26]. The surfactant ratio was set at about 20% w/V, since Akram et al.[26] demonstrated it was able to provide the formation of homogeneous droplets with low PDI values. Basing on the consideration that a mixture of hydrophilic and lipophilic surfactants provides a better stabilization of the MEs compared to a single surfactant[36], we chose to add both surfactant (Labrafil® or Span® 80) and co-surfactant (Kolliphor® or Tween® 80), in different combinations. As a consequence, the 8 primary W/O MEs produced, were analyzed through PCS (Table 1). Among the prepared ME2, only ME2-A (produced with Labrafil® and Kolliphor® as surfactant mixture, and IPM as oil) showed small droplets size and great homogeneity, thus it was selected for further studies.

TABLE 1 SHOULD BE INSERTED HERE

3.1.2 ME2-A stirring time optimization

Stirring rate was reported to have a great influence on the mean size of the obtained droplets, as elaborated on by Akram et al.[26], whose studies demonstrated that 500 rpm was the optimal stirring rate value: lower stirring values were not able to provide sufficient energy to the emulsive system, causing instability; on the other hand, even if high stirring rates could reduce the time needed for the obtainment of the emulsion, the fast agitation could induce coalescence of the droplets limiting the stabilization due to the surfactant[26]. Moreover, it was reported that also stirring time has a great influence on droplet size and homogeneity, and – in the situation of a multiple emulsions – on the inner phase droplets[37]. In particular, considering 500 rpm stirring, it was demonstrated that an increase in stirring time from 15 min to 45 min causes an increment in

the droplets mean diameter[37]. For this reason, in our studies 500 rpm was set at the stirring rate, while different stirring times were screened on ME2-A; the obtained droplet sizes and PDI values are reported in Supplementary Figure 1. Based on the obtained results, combining both lower droplets size with good homogeneity values, 30 min was selected as the optimal stirring time.

3.2. Preformulative studies and characterization of the triphasic MEs

3.2.1. ME3 optimization and characterization

In order to obtain secondary triphasic W/O/W microemulsion (ME3), the selected primary W/O biphasic ME2-A was inverted in TRIS using the PIT technique, which allows to avoid the use of organic solvents, being an eco-friendly preparation method, and permits to potentially encapsulate molecules which could be degraded by high-energy techniques[23,24]. Since it is well known that phase ratio and amounts of surfactants are crucial parameters in the determination of droplet size because of aggregation phenomena[38], three ME2-A to TRIS ratios were screened, adding different percentages of Tween® 80, which was selected for its proper HLB value and because it was found to help obtaining O/W microemulsions with small droplet diameter (<85 nm)[39]. All the produced formulations were analyzed using PCS in order to measure mean droplet size and PDI values. The obtained results, reported in Figure 1, highlighted an initial increase in ME3-A droplet size after the addition of a minimal amount of surfactant (1% or 2% w/V), compared to the sample without Tween® 80. These results could be related to the insufficient amount of surfactant at the droplet interface, which enhanced coalescence. Increasing amounts of Tween® 80 led to the formation of smaller droplets, as expected, with lower sizes at higher Tween® 80 concentrations. Basing on these considerations, ME3-A diluted in TRIS buffer at 1:3 with 4% w/V of Tween® 80 was selected as the optimal formulation and subjected to further studies, having droplets size adequate for the nasal administration and slightly negative (c.a. -8 mV) ZP values (Table 2).

FIGURE 1 SHOULD BE INSERTED HERE

3.2.2. Surface modification and fluorescein loading

As reported in literature, a neutral or negative ZP value could be suitable for both nose-to-brain and local nasal administration, while the surface modification with a cationic agent could enhance interactions with mucin[40]. Thus, aiming to increase the residence time on the nasal mucosa, the cationic surfactant DDAB was added to the primary W/O biphasic ME2-A, thus obtaining ME2+A, which was subsequently converted in the secondary triphasic W/O/W microemulsion (ME3+A), as previously described for the neutral ME. The addition of DDAB slightly decreased the mean droplet size of ME3+A compared to ME3-A; this phenomenon was already reported for another microemulsion system, where the addition of different amount of the cationic surfactant cetyltrimethylammonium bromide (CTAB) caused a variation of ME droplet size[41]. Moreover, pH and osmolality of nasal formulations have to be adequate in order to avoid mucosal toxicity. A wide pH range is well-tolerated by nasal mucosa[42], even if values close to physiological ones (4.5–6.5) are optimal; on the other hand, pH values lower than 3 or higher than 10 can promote the occurrence of irritation phenomena[43]. Osmolality also represents a crucial parameter in nasal administration, which should be isotonic, thus inhibiting ciliary activity[43], or hypotonic,, increasing permeation through nasal mucosa[44]. Based on these considerations, the results obtained from the technological characterization describe all the obtained formulations as suitable and promising for a potential nasal administration.

TABLE 2 SHOULD BE INSERTED HERE

3.2.3. Accelerated stability studies

Both blank ME3-A and ME3+A showed a good stability during 6 months-storage time in accelerated conditions (Figure 2), which is representative of 1 year stability at 25±2°C and 75±5% RH[25]. Cationic ME3+A demonstrated higher stability, with a minimal variation in droplets size, significant (*$p<0.05$) only at two timepoints. This result was expectable since net positive or negative ZP values are able to guarantee a sufficient droplets repulsion, which prevents the occurrence of instability phenomena[45]. However, even if the almost neutral superficial charge of the ME3-A system induced some variation in droplets size during storage, which is significant (****$p<0.0001$) from 3 months of storage, the droplets diameters remained into the microemulsion size range (<200 nm)[6].

FIGURE 2 SHOULD BE INSERTED HERE

3.3. *In vitro* studies

3.3.1. Mucoadhesion studies

The key event in the delivery and absorption of a drug after nasal administration is its passage through the mucus. Mucin, which is formed from mucus, is a protein that has the potential to bind solutes and thus plays a critical role in the drug diffusion process[46]. Factors related to the physical-chemical properties of the formulation and to the nasal cavity may affect the nasal absorption of the drug. One of the main issues hampering drug absorption after nasal administration is the rapid mucociliar clearance that reduces the capacity of drug absorption and its bioavailability[47]. Thus, during the design of a formulation intended for the nasal route, it is of great importance to evaluate its mucoadhesive strength and its potential interaction with the main glycoprotein of the nasal mucosa. In light of these considerations, mucoadhesive properties of ME3-A and ME3+A were evaluated after interaction with mucin by assessing the variation in the ZP values (Figure 3a) and in the turbidity at 650 nm (Figure 3b).

FIGURE 3 SHOULD BE INSERTED HERE

ZP measurements is a common method to investigate the mucoadhesive properties of several matrix and to evaluate the interactions of nanocarriers with mucin[48]. Significant variations in ZP values were reported for both MEs at all considered time points. In particular, upon addition of ME3-A and ME3+A to mucin, the negative ZP of mucin (~ –5 mV) was significantly shifted to more neutral values (Figure 3a) (***$p<0.001$ at T0), and this effect was even more significant (****$p<0.0001$ at T0) for mucin mixture with ME3+A probably due to the occurrence of stronger interaction between the deprotonated carboxylate groups (sialic acid) and ester sulfates at the terminus of some sugar units on mucin glycoproteins and the DDAB tertiary amino group present on the surface of positively charged droplets. Anyway, the significant variation in ZP values, reported for ME3-A since the first analyzed timepoint (***$p<0.001$), suggests its interaction with mucin, thus it also possesses interesting mucoadhesive properties.
Furthermore, absorbance measurement analysis was performed to confirm sample-mucin interaction. The mucoadhesive interaction between MEs and mucin results in the adsorption of the mucin around the surface of the droplets with a consequent slight aggregation that can be detected as an increase in UV absorbance[49]. The turbidity of ME3-A and ME3+A /mucin dispersions (Figure 3b) was significantly higher than the turbidity of the mucin dispersion itself at all the time points considered for both formulations (****$p<0.0001$), thus suggesting that interaction phenomena occurred for both ME3-A and ME3+A.
The reported interactions of ME3-A and ME3+A with mucin could promote the residence time of the formulations at the site of administration, offering an easily accessible route to the immune system inducing both mucosal and systemic immunity.

3.3.2. Fluorescein loading and release

In this preliminary study, a hydrophilic model drug was selected in order to evaluate the preliminary physico-chemical and technological features of the developed microemulsions, thus to select the best parameters to obtain the ideal formulation for the intended administration route. To analyze the behavior of the carrier itself, basing on literature findings, fluorescein sodium salt was selected[50–52], resulting to be compatible with the preparation technique, as reported in literature[53] for its stability at neutral pH and no occurrence of thermic degradation up to 70°C for 12 days. In order to analyze the behavior of a potential encapsulated drug, fluorescein was selected as hydrophilic model drug and loaded into the inner water phase of ME2-A and ME2+A, which were subsequently added of 4% of Tween® 80 and diluted 1:3 in TRIS, thus obtaining the loaded secondary triphasic W/O/W microemulsions F-ME3-A and F-ME3+A. The physical-chemical and technological features of both the loaded ME3s resulted to be unaltered compared to the respective blank samples (Table 2). A slight not significant increment of Z-ave and PDI after encapsulation was reported and could be related to the steric hindrance of the probe, which could enlarge the inner aqueous core space[54]. pH and osmolality values were not affected by the addition of the fluorescein.
In vitro release of the probe from the two loaded ME3s (Figure 4) highlighted a different behavior for F-ME3-A and F-ME3+A. In particular, F-ME3-A performed a sustained release, with the 50% of drug released in the first 2h, reaching the 100% at 24h. F-ME3+A, on the other hand, showed a slower release, with 50% of fluorescein released after 8h from the beginning of the experiment, up to 60% after 24h. Considering the anionic nature of fluorescein sodium salt, characterized by the presence of two O^- residues, it is possible that, in the microemulsion F-ME3+A, an electrostatic interaction occurs with the cationic DDAB and the probe, thus slowing down the release, as previously reported in literature[55]. It is worth to note that the higher sustained drug release provided by F-ME3-A could be an advantage due to its reduced retention time on the nasal mucosa, compared to the positively charged system, thus both formulations show physical-chemical and technological properties that could be exploited for a potential therapy thought nasal administration.

FIGURE 4 SHOULD BE INSERTED HERE

3.3.3. Cytotoxicity evaluation

Formulations biocompatibility represents an important prerequisite for their pharmaceutical application. Considering the features of the nasal administration route, the nasal tissue is the first body's barrier and its integrity must be preserved in order to perform its function properly[28]. The MTT assay, an easy and common test for cytotoxicity studies, was chosen to assess the biocompatibility of the secondary triphasic W/O/W microemulsions (ME3-A and ME3+A). The cytocompatibility of the secondary triphasic microemulsions was assessed by MTT on human airway epithelial (Calu-3) and human fibroblast (HFF1) cells lines, selected as a suitable *in vitro* model of the nasal milieu for the development of nasal products[27].The results of the cell viability tests are reported in Figure 5.

FIGURE 5 SHOULD BE INSERTED HERE

ME3-A formulation showed to be safe on Calu-3 after 0.5, 1 and 2h of treatment for all the tested concentrations. At the highest tested concentration (8.81 mg/ml), ME3-A resulted in a small reduction in the cellular viability of approximately 16% after 3h of treatment, compared to the untreated cells (Figure 5a) (significance to CTRL– *p<0.05). This is an interesting result, since it has been reported that many intranasal formulations show a clearance half-life of about 15 min[56]. Therefore, this observed biocompatibility up to 3h, along with a good mucoadhesion, may ensure efficient drug penetration. The cytotoxicity studies on the HFF1 human fibroblasts at 0.5, 1, and 2h showed the occurrence of a slight decrease in cell viability in the treatment with the neutral ME3-A, with percentages of viability very similar to each other, thus independent of both concentration and time (significance to CTRL– ***p>0.001 for the higher concentration at all timepoints). However, after 3h of treatment, ME3-A significantly (****p<0.0001 to CTRL–) reduced the cell viability in a concentration-dependent manner, with around 58% of cells viable at the highest concentration tested (Figure 5b). In the case of positively charged ME3+A, a marked reduction of cell viability was observed (significance to CTRL– ****p<0.0001), in a concentration- and time-dependent manner, in Calu-3 cells (Figure 5c) and in a greater extent in HFF1 cells (data not shown). The cytotoxic effect of ME3+A agrees with previous studies reporting a higher toxicity of positively charged surfactants on cells and tissues[57–60].
As regard the effect on fibroblasts, it should be noted that this *in vitro* monolayer model does not reflect *in vivo* conditions, as the fibroblasts are located below the nasal epithelium layer[61]. Therefore, they would not be in direct contact with the formulation, but – depending on the mode of absorption through the nasal epithelium – they would be exposed to more dilute formulations or individual components. In addition, the nasal stromal tissue is highly vascularized[62], therefore the residence time of the formulation would be much shorter than the tested time points.

4. Conclusions

The initial screening on raw materials resulted in the selection of Kolliphor® and Labrafil® as surfactants for the preparation of the primary biphasic W/O ME, which resulted optimal with the use of IPM as oily phase. Both neutral and cationic secondary triphasic W/O/W ME3-A and ME3+A, obtained by dilution 1:3 with TRIS and the addition of a proper amount of Tween 80, demonstrated to have adequate physical-chemical and technological features for the nasal administration, with a good long-term stability. Considering its higher cytocompatibility, ME3-A represents the optimal candidate for the nasal mucosal administration of hydrophilic drugs, due to its ability to adequately interact with the site of administration providing a sustained drug release. Moreover, the employment of PIT technique resulted to represent a valid strategy to avoid the use of high-energy techniques as well as organic solvents, considering the possible degradation of the potential hydrophilic molecules to be delivered (such as vaccines and proteins). Finally, the triphasic microemulsion developed represents a potential valid strategy for the combined delivery of hydrophilic and lipophilic molecules, with the advantage to improve the carrier stability.

Declarations of interest: none

Funding: Cinzia Cimino was supported by the PhD program in Biotechnology, XXXVI cycle, University of Catania. This work was supported by a grant from the Italian Ministry of Research [Grant PRIN 2017 #20173ZECCM - Tracking biological barriers to antigen delivery by nanotechnological vaccines (NanoTechVax)].

Acknowledgments: The authors are grateful to NANOMED, Research Centre for Nanomedicine and Pharmaceutical Nanotechnology from the University of Catania for the technical assistance.

Conflicts of Interest: The authors declare no conflict of interest.

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Figures Captions

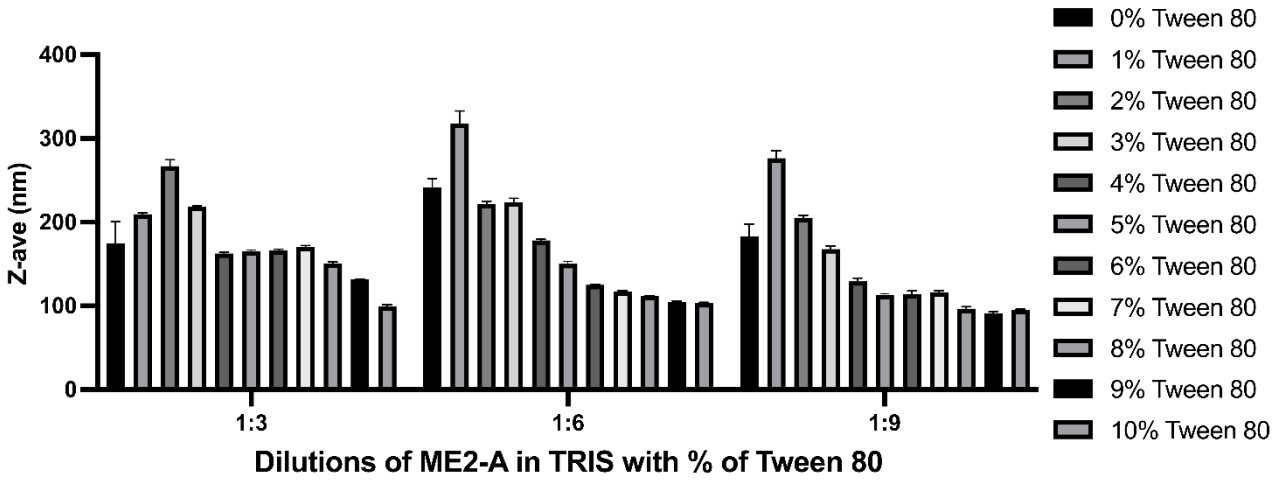

Figure 1. Mean droplet size ME3-A, with different dilutions of ME2-A in TRIS and different amounts of Tween® 80. Values are reported as mean of at least 3 measurements ± SD.

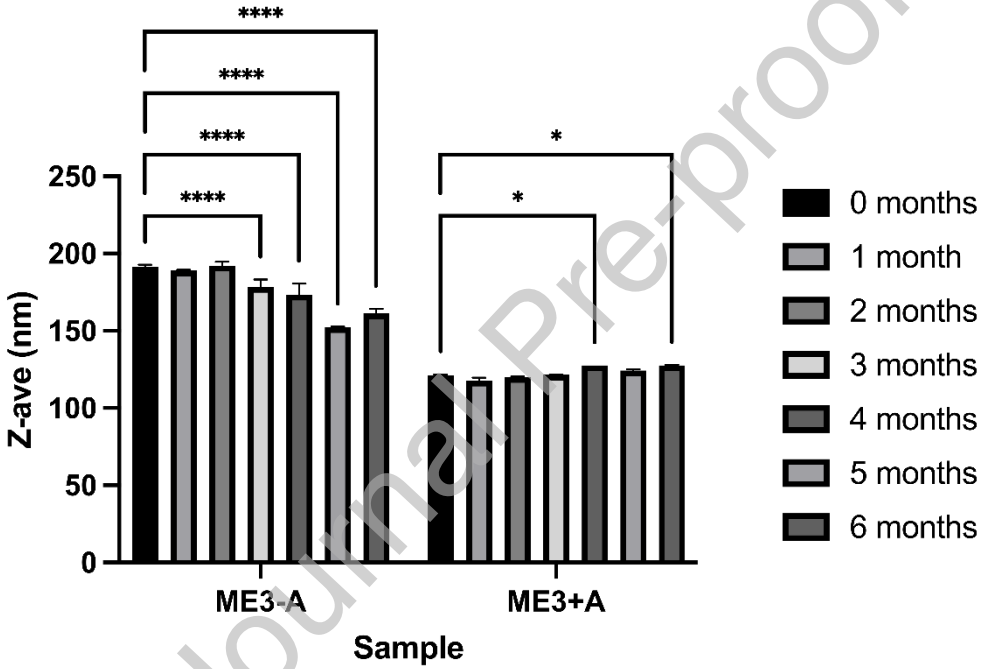

Figure 2. Z-ave (nm) of unloaded ME3-A and ME3+A stored in accelerated conditions at 40±2°C and 75±5% RH, for 6 months. Values are reported as mean of at least 3 measurements ± SD. Significance was set at *p≤0.05; ****p≤0.0001.

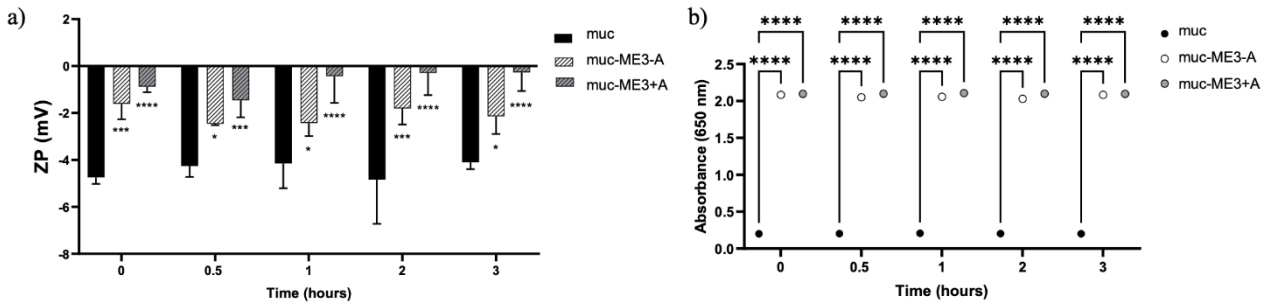

Figure 3. (**a**) Zeta potential values (ZP) of mucin (muc) before 0 and after 0.5, 1, 2 and 3h of incubation with ME3-A and ME3+A (muc-ME3-A; muc-ME3+A) at 37°C. Significance was set at ****p≤0.0001; ***p≤0.001; *p≤0.05. (**b**) *In vitro* assessment of ME3-A and ME3+A /mucin interactions by turbidimetric assay at 650 nm. Significance was set as **** p≤0.0001 for all samples vs muc.

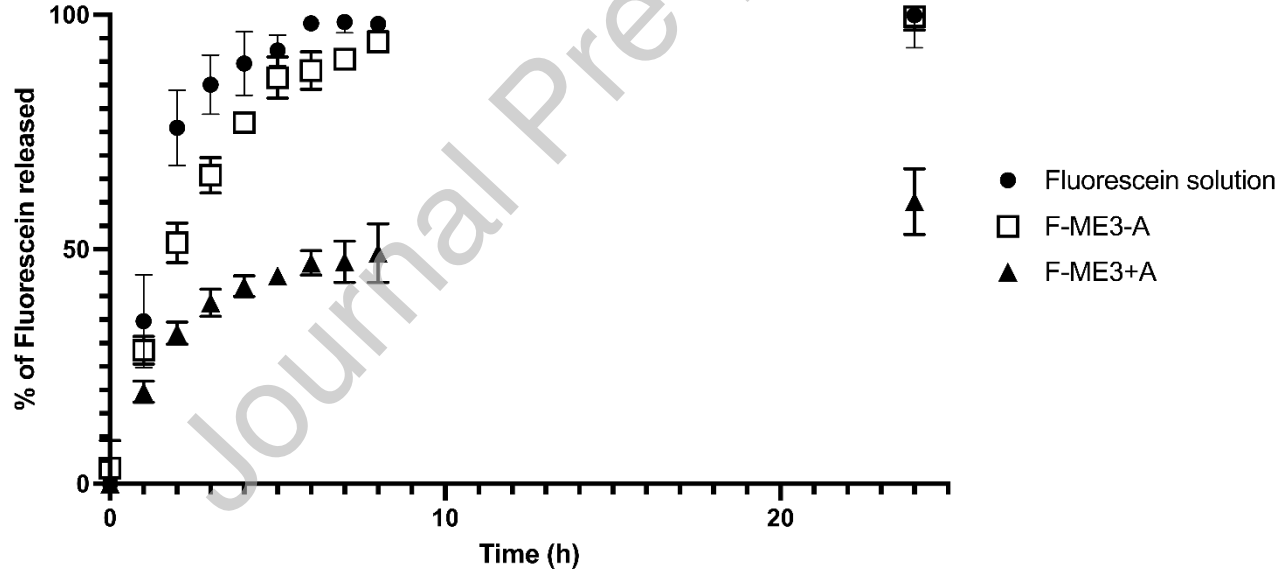

Figure 4. Fluorescein release from F-ME3-A and F-ME3+A, compared to free fluorescein solution. Values are reported as mean of at least 3 measurements ± SD.

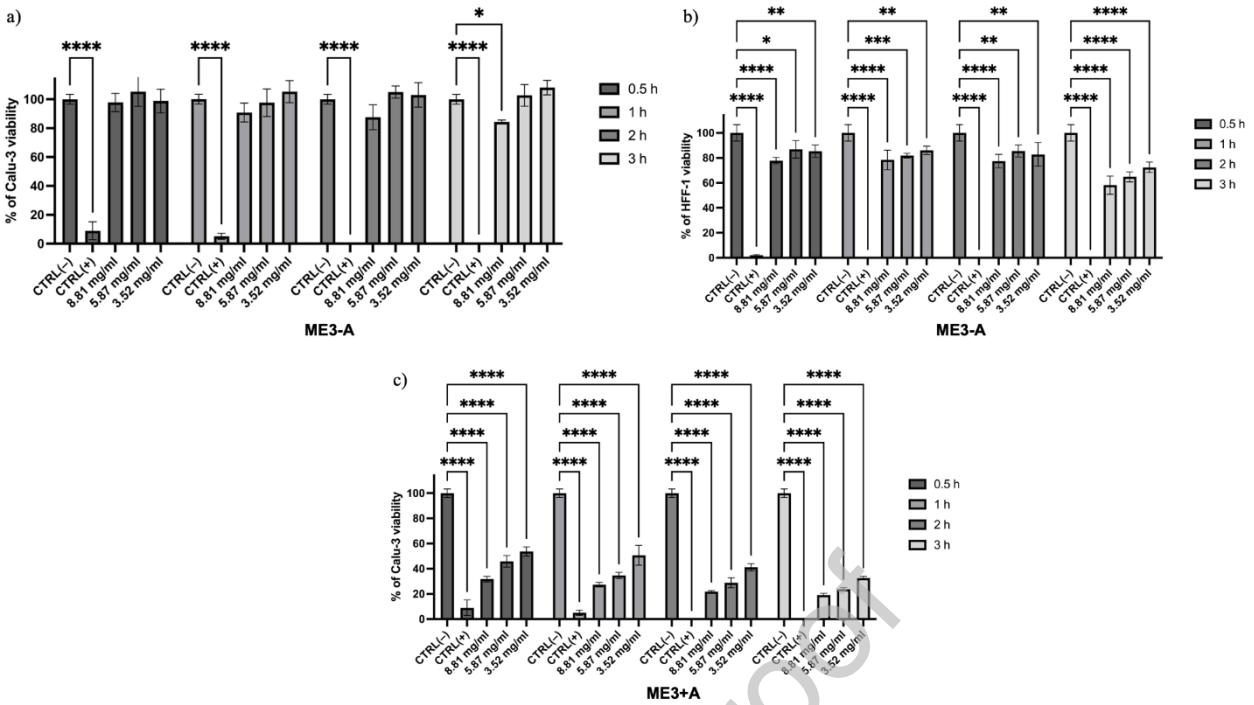

Figure 5. Cell viability of Calu-3 and HFF1 cell lines treated with different concentrations of microemulsion's at different time points (0.5, 1, 2 and 3h): neutral unloaded ME3-A treatment with Calu-3 (**a**); neutral unloaded ME3-A treatment with HFF1 cell lines (**b**); cationic unloaded ME3+A treatment with Calu-3 (**c**). Graphs display cells viability expressed as a percentage in comparison to untreated cells. Values are reported as mean ± SD of at least 3 experiments conducted in quadruplicates. Significance *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001 versus untreated CTRL–. Significance versus SDS 0.2% CTRL+ was not reported in the graphs (significant for ****p≤0.0001 for all the concentrations at all timepoints).

Supplementary Figure 1. Mean droplets size (Z-ave) and polydispersity index (PDI) primary W/O biphasic microemulsion ME2-A subjected to different stirring time.

Tables

Table 1. PCS measurements of mean droplets size (Z-ave) and polydispersity index (PDI) of primary W/O microemulsions ME2 (from A to H). Values are reported as mean of at least 3 measurements ± SD. (nd: data not measured).

Sample	Z-ave (nm) ± SD	PDI ± SD
ME2-A	89.24 ± 6.37	0.188 ± 0.074
ME2-B	180.40 ± 43.97	0.588 ± 0.368
ME2-C	nd	nd
ME2-D	425.10 ± 49.14	0.874 ± 0.128
ME2-E	299.60 ± 28.36	0.390 ± 0.468
ME2-F	998.20 ± 452.30	0.609 ± 0.208
ME2-G	nd	nd
ME2-H	nd	nd

Table 2. Characterization of unloaded ME3-A and ME3+A, and fluorescein-loaded F-ME3-A and F-ME3+A. Mean droplet size (Z-ave), PDI, zeta potential (ZP), pH and osmolality are reported, and each value is the mean of at least three measurements ± SD. Statistics were calculated comparing the two blanks and each loaded with the respective blank. Significance was set at ***p≤0.001; ****p≤0.0001.

Sample	Z-ave (nm) ± SD	PDI ± SD	ZP (mv) ± SD	pH ± SD	Osm (mOsm/kg) ± SD
ME3-A	172.50 ± 13.15	0.301 ± 0.018	-8.00 ± 0.39	6.28 ± 0.01	0.215 ± 0.001
F-ME3-A	197.40 ± 36.35	0.321 ± 0.062	-7.99 ± 0.46	6.34 ± 0.01	0.238 ± 0.006
ME3+A	118.30 ± 16.40****	0.452 ± 0.062	+19.10 ± 0.07***	6.21 ± 0.01	0.231 ± 0.005
F-ME3+A	142.40 ± 4.95***	0.477 ± 0.045	+20.50 ± 1.98	6.23 ± 0.01	0.218 ± 0.001

Declaration of interests

☒ **The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.**

☐**The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:**

Graphical Abstract