Regular Article

Zeta potential changing nanoemulsions based on a simple zwitterion

Faezeh Sharifi, Mansour Jahangiri, Imran Nazir, Mulazim Hussain Asim, Pedram Ebrahimnejad, Andrea Hupfauff, Ronald Gust, Andreas Bernkop-Schnürch

Hypothesis: Simple zwitterions used as auxiliary agents might have the potential to change the zeta potential of oil-in-water (o/w) nanoemulsions on the mucosa.

Experiments: The zwitterion phosphorylated tyramine (p-Tyr) was synthesized by phosphorylation of Boc-tyramine (Boc-Tyr) using phosphoryl chloride (POCl₃). It was incorporated with 2% (m/v) in a self-emulsifying lipophilic phase comprising Captex 35, Cremophor EL, Capmul MCM and glycerol 85 at a ratio of 30:30:30:10 v/v. Phosphate release and resulting change in zeta potential were evaluated by incubating p-Tyr containing nanoemulsion with isolated intestinal alkaline phosphatase (AP). Mucus permeating behavior was evaluated across mucus obtained from porcine small intestinal mucosa. Subsequently, cellular uptake studies were accomplished on Caco-2 cells.

Findings: The p-Tyr loaded nanoemulsion exhibited a mean droplet size of 43 ± 1.7 nm and zeta potential of −8.40 mV. Phosphate moieties were rapidly cleaved from p-Tyr loaded nanoemulsions after incubation with isolated AP resulting in a shift in zeta potential from −8.40 mV to +1.2 mV. p-Tyr loaded...
nanoemulsion revealed a significantly ($p \leq 0.001$) improved mucus permeation compared to the same nanoemulsion having been pre-treated with AP. Cellular uptake of the zeta potential changing oily droplets was 2.4-fold improved. Phosphorylated zwitterions seem to be an alternative to cationic surfactants and considered as promising auxiliary agents for zeta potential changing nanoemulsions.

© 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).

1. Introduction

To overcome the anionically charged mucus gel layer on the one hand and to provide a positive charge on the cell membrane for an improved cellular uptake on the other hand, the approach of zeta potential changing nanocarriers was pioneered [1]. Nanocarriers displaying a negative zeta potential due to phosphate moieties on their surface were shown to rapidly permeate the negatively charged mucus gel layer covering mucosal membranes. When these phosphorylated nanocarriers reach the epithelium, the anionic phosphate moieties are cleaved off by alkaline phosphatase (AP) expressed on the surface of epithelial cells resulting in a shift in zeta potential to positive values and consequently in an improved cellular uptake. Among zeta potential changing nanocarriers, polymeric nanoparticles [2], micelles [3] and o/w nanoemulsions [4] have been developed so far and first in vivo studies provide evidence for the great potential of this concept [5]. Nanoemulsions seem to be most attractive from the industrial point of view as their scale up is comparatively simple and sophisticated production technologies are not required [6,7]. Suchaoin et al. designed the first zeta potential changing nanoemulsion exhibiting a zeta potential change from just $-1$ mV to $+1$ mV [8].

Up to date, all zeta potential changing nanoemulsions contain a cationic surfactant after phosphate cleavage being responsible for a positive zeta potential. Lipophilic cationic compounds, however, are of toxicological concern and even the use of well-established preservatives such as benzalkonium chloride or cetrimonium bromide seem to be problematic as comparatively high amounts of these agents are needed for zeta potential changing systems [8].

Alternatives to cationic surfactants would allow to overcome this shortcoming.

It was therefore the aim of this study to investigate the potential of an alternative to cationic surfactants. In order to keep the auxiliary agent being responsible for the change in the zeta potential concentrated on the oily droplets, however, such alternatives have to exhibit poor solubility in aqueous media. So far, this was achieved by a pronounced lipophilic structure. As many zwitterions are also poorly soluble in aqueous media even without such a pronounced lipophilic structure, they might be such an alternative. To verify this working hypothesis, phosphorylated tyramine was chosen since tyramine is an endogenous compound occurring widely in food such as meat, fish and aged cheese [9,10]. Herein, we synthesized p-Tyr, a simple zwitterion, and incorporated it into nanoemulsions in order to evaluate its impact on change in zeta potential due to phosphate cleavage by AP.

2. Materials and methods

2.1. Materials

Boc-tyramine (Boc-Tyr) was purchased from TCI Chemicals (Germany). Phosphoryl chloride (POCl₃), deuterium chloride (DCl) solution, alkaline phosphatase from bovine gut mucosa containing 7165 unit·mg⁻¹ protein (19.4%), 4-(2-hydroxyethyl)-1-piper-azineethanesulfonic acid (HEPES ≥ 99.5%), sulfuric acid (H₂SO₄), Triton™ X-100, malachite green (MLG) oxalate salt (≥90%), ammonium molybdate tetrahydrate (≥83%), potassium phosphate monobasic (KH₂PO₄ ≥ 99.5%), l-cysteine HCl, 2,4,6-trinitrobenzene sulfonic acid (TNBS), Tween 80, propylene glycol, fluorescein diacetate and phosphatase inhibitors cocktail II were obtained from Merck (Austria). Deuterated methanol was purchased from Eurisotop (France). Anhydrous pyridine was obtained from Alfa Aesar (Germany). Cremophor EL, Capmul MCM and Captex 355 were provided by Abitech (Germany). All chemicals were used without further purification.

2.2. Methods

2.2.1. Synthesis and characterization of phosphorylated tyramine (p-Tyr)

p-Tyr was synthesized as illustrated in Fig. 1. Briefly, 121 mg of Boc-Tyr (0.51 mmol) were dissolved in 0.2 mL (2.5 mmol) of anhydrous pyridine. Under cooling with an ice/salt bath, 1 mL (10.96 mmol, 1680 mg) of POCl₃ was slowly added and led to a white precipitate. The reaction mixture stirred for 1 h under ice cooling and further 24 h at room temperature. Excess of pyridine and POCl₃ was removed in vacuo. The crude product was hydrolyzed with ice under ice cooling and heated to 60 °C for 2 h. The water was distilled off and the precipitate was washed with dichloromethane, followed by dichloromethane and 10% methanol (50 mL). The obtained solid was rinsed with 300 mL of methanol. The colorless powder was dried over silica gel in a desiccator overnight.

Yield: 80 mg (0.37 mmol, 73% as colorless powder).

HR-MS: [M+H]+ calc. 218.0577, measured 218.0552; [M−=NH₂] calc. 201.0311, measured 201.0289.

FT-IR: 3000–2500 br, 2321 w, 1609 m, 1506 m, 1219 m, 1144 m, 1102 m, 1023 m, 944 s, 531 s.

Fig. 1. Synthetic pathway for the preparation of phosphorylated tyramine (p-Tyr).
\[^{1}\text{H}\] NMR (400 MHz, CD\textsubscript{3}OD/DCl): \(\delta = 2.99–3.05\) (m, 2H, CH\textsubscript{2}), 3.15–3.22 (m, 2H, CH\textsubscript{2}NH\textsubscript{2}), 7.19 (m, 2H, ArH-2 and ArH-6), 7.33 (m, 2H, ArH-3 and ArH-5).

\[^{13}\text{C}\] NMR (101 MHz, CD\textsubscript{3}OD/DCl): \(\delta = 32.3, 40.6, 120.35, 120.40, 129.8, 133.3, 150.27, 150.34\).

\[^{31}\text{P}\] NMR (162 MHz, CD\textsubscript{3}OD/DCl): \(\delta = -5.49\).

p-Tyr was characterized by high-resolution mass spectrometry (HR-MS), infrared (IR) and \(^{1}\text{H}, ^{13}\text{C}, ^{31}\text{P}\) and \(^{31}\text{P}-^{1}\text{H}\) long range correlation nuclear magnetic resonance (NMR) spectroscopy. High resolution mass spectra: Thermo Fisher Orbitrap Elite, equipped with an ESI ion source. FT-IR spectra: Bruker ALPHA FT-IR apparatus equipped with a Platinum ATR module with 24 scans at a resolution of 4 cm\(^{-1}\) in the wavenumber range from 4000 to 400 cm\(^{-1}\).

NMR spectra: Bruker Avance 4 Neo spectrometer (\(^{1}\text{H}: 400.13 \text{ MHz}, ^{13}\text{C}: 100.62 \text{ MHz}, ^{31}\text{P}: 161.99 \text{ MHz})). The center of the solvent signal was used as internal standard. Samples were dissolved in 0.6 mL of methanol \(d_4\) or in methanol \(d_4/DCl\) (addition of 0.06 mL DCl solution (35% in D\textsubscript{2}O)).

2.2. Development and characterization of nanoemulsions

In order to develop nanoemulsions, excipients as listed in Table 1 were mixed in various ratios whereby solid and semisolid excipients were melted prior to use. Tween 80 and Cremophor EL were used as self-emulsifying agents, Labrafil and Capmul MCM as co-surfactants, Captex 355, Peceol and ethyl oleate as oily vehicle and Triacetin, glycerol and propylene glycol as solvents. A single homogeneous lipophilic phase was obtained by mixing the excipients of each formulation by ultrasonication for 10 min followed by vortex mixing at 1000 rpm and 50 \(^\circ\)C. p-Tyr (2% m/v) was incorporated in this lipophilic phase via ultrasonication for 10 min followed by shaking for 2 h at 1000 rpm and 50 \(^\circ\)C. To confirm the entire incorporation of p-Tyr, the lipophilic phase was centrifuged at 12,100 g for 10 min and evaluated visually. The lipophilic phase was emulsified in a ratio of 1:100 (v/v) with 100 mM HEPES buffer containing 0.2 mM ZnCl\(_2\) and 5 mM MgCl\(_2\) pH 7.0. The preparation of nanoemulsions is illustrated in Fig. 2. Blank as well as 2% (m/v) p-Tyr loaded oily droplets were characterized by dynamic light scattering with Zetasizer Nano-ZS (Malvern Instruments, UK).

2.2.3. Determination of distribution coefficient

\((\log D^\text{lipophilic phase/release medium})\)

The release of the zwitterion from nanoemulsion was evaluated by measuring \(\log D^\text{lipophilic phase/release medium}\) value \([13]\). The solubility of the zwitterion in the lipophilic phase of the nanoemulsion and in the release medium was evaluated in a separate manner. Increasing concentrations of p-Tyr were dissolved in the lipophilic phase of nanoemulsions while shaking at 500 rpm and 37 \(^\circ\)C until first turbid dispersions remained indicating that the maximum solubility has been exceeded. Solubility of the zwitterion in release medium was determined by measuring the absorbance of p-Tyr at wavelength of 220 nm using spectrophotometer. For this, 1 mg of p-Tyr was dissolved in 1 mL of release medium while shaking at 400 rpm and 37 \(^\circ\)C for 6 h. Demineralized water and 100 mM HEPES buffer containing 0.2 mM ZnCl\(_2\) and 5 mM MgCl\(_2\) (HB) pH 7.0 were used as release medium. \(\log D^\text{lipophilic phase/release medium}\) was measured using Eq. (1):

\[
\begin{array}{c}
\text{Table 1} \\
\text{Composition of nanoemulsion formulations. Values are indicated in percent (v/v).}
\end{array}
\]

| Formulations | Capmul MCM | Peceol | Captex 355 | Ethyl oleate | Labrafil | Cremophor EL | Tween 80 | Triacetin | Glycerol 85 | Propylene glycol |
|--------------|------------|--------|------------|--------------|----------|--------------|----------|-----------|-------------|-----------------|
| FI           | –          | 10     | –          | –            | 40       | 40           | –        | –         | –           | 10              |
| FII          | 30         | –      | 30         | –            | –        | 30           | 50       | 10        | –           | 10              |
| FIII         | –          | –      | 30         | –            | –        | –            | 50       | 10        | 10          | –               |
| FIV          | 20         | –      | 30         | –            | –        | –            | 30       | 10        | 10          | –               |
| FV           | 30         | –      | 20         | –            | –        | 30           | –        | –         | –           | 20              |

Fig. 2. Illustration of the method used to prepare p-Tyr loaded nanoemulsions.
2.2.4. Phosphate release studies

Phosphate release from nanoemulsions loaded with p-Tyr was evaluated after incubation with isolated AP as described previously [4]. Nanoemulsion loaded with p-Tyr was diluted 1:100 with 100 mM HB pH 7.0. For this, 100 μL of isolated AP yielding an enzyme activity of 10 U/mL was added to 10 mL of p-Tyr loaded nanoemulsion. The enzymatic reaction was allowed to proceed for 6 h under constant shaking at 500 rpm and 37 °C. At predetermined time points (0, 1, 2, 3, 4, 5 and 6 h), 50 μL of samples were removed and enzymatic reaction was stopped by adding 5 μL of 3.6 M H2SO4. Nanoemulsion loaded with p-Tyr having been incubated without AP served as control. The concentration of cleaved phosphate was measured by malachite green (MLG) assay. MLG reagent was prepared by dissolving 0.15% m/v MLG oxalate salt in 3.6 M H2SO4 followed by addition of 400 μL of aqueous solution of 11% m/v Triton™ X-100. Then, 6 mL of aqueous solution of ammonium molybdate (8% m/v) was added dropwise to 10 mL of above-mentioned solution under vigorous stirring. Each test sample was homogenized with 100 μL of MLG reagent. The absorbance was measured at a wavelength of 630 nm using a microplate reader. The amount of cleaved phosphate was determined by means of a calibration curve having been established with increasing concentrations of KH2PO4 [14].

Cleavage of phosphate substructures from p-Tyr loaded nanoemulsions was further evaluated on Caco-2 cells. Cells were seeded into a 24-well plate at a density of 2.5 × 10⁴ cells per well and cultured in minimum essential medium (MEM) augmented with 10% (v/v) fetal bovine serum (FBS), 2 mM l-glutamine and 1% (v/v) streptomycin-penicillin incubated at 37 °C in an environment of 95% relative humidity and 5% CO2. Cell culture media was replaced every two days until a confluent monolayer was provided. After 3 h of incubation, aliquots of 100 μL of samples were withdrawn and absorbance measurements at 420 nm were performed utilizing a microplate reader. The amount of primary amino groups was determined using a calibration curve having been established with increasing concentrations of L-cysteine HCl. As control, samples was incubated without adding the enzyme.

2.2.7. Impact on viability of Caco-2 cells

The impact of p-Tyr and p-Tyr loaded nanoemulsions on Caco-2 cells viability was assessed using resazurin assay [4]. Briefly, Caco-2 cells were cultured into a 24 well plate at a density of 2.5 × 10⁴ cells per well in MEM at 37 °C in an environment of 95% relative humidity and 5% CO2 as described in 2.2.4.2. Cell culture media was supplantated every two days until a continuous monolayer was provided. Samples in a concentration of 0.25% (m/v) p-Tyr were prepared in a solution of PEG 300:DMA 1:1 (v/v) and diluted 1:50 with 25 mM HEPES buffered saline (HBS). Hence, 2% (m/v) p-Tyr loaded nanoemulsion was diluted 1:500 and 1:100 in HBS. Moreover, HBS was utilized as a positive control, whereas 0.5% (m/v) Triton™ X-100 solution served as negative control. Before starting the experiment, cells were washed twice with pre-heated HBS buffer at 37 °C. In the following, 500 μL of positive control, negative control, samples of 0.25% p-Tyr and p-Tyr loaded nanoemulsions were added in triplicate. After incubation for 6 h under the abovementioned conditions, samples were removed. Thereafter, cells were washed two times with pre-heated buffer and medium was replaced by 500 μL of 2.2 mM resazurin solution. After 3 h of incubation, aliquots of 100 μL were transferred to a 96-well black microtiter plate and fluorescence intensity was measured at an excitation and an emission wavelength of 540 nm and 590 nm, respectively. Cell viability was calculated using Eq. (2):

\[
\text{Cell viability} = \left( \frac{\text{Sample fluorescence} - \text{negative control fluorescence}}{\text{Positive control fluorescence} - \text{negative control fluorescence}} \right) \times 100
\]

2.2.8. Mucus permeation studies

Porcine small intestinal mucosa was obtained from a local abattoir while stored on ice during transfer to the laboratory. The sections of the small intestine were cut longitudinally and mucus was scraped off from the intestine. The obtained mucus was purified via adding 5 mL of sodium chloride (0.1 M) to each 1 g of mucus being stirred for 1 h at 4 °C. After centrifugation at 11,246g for 2 h at 4 °C the supernatant was removed and purified mucus was stored at −20 °C until used.

The permeation of p-Tyr loaded nanoemulsion through mucus was estimated by Transwell® method as described previously [15]. For this purpose, 50 mg of purified mucus was applied on TransCert inserts occupying a surface area of 33.6 mm² and a pore diameter of 3.0 μm (Greiner Bio-One GmbH, Kremsmünster, Austria) being placed in a 24-well plate. In order to investigate the permeation of oily droplets through mucus, lipophilic phase of nanoemulsion was labeled by adding 0.5% (v/v) fluorescein diacetate solution (1 mg/mL in DMSO). Afterward, 250 μL of formulation FII loaded with p-Tyr and fluorescein diacetate having been diluted 1:100 in 100 mM HEPES buffer pH 7.4 was added into the donor chamber. The acceptor chamber was filled with 500 μL loaded nanoemulsions having been diluted 1:100 in 100 mM HB pH 7.0 was incubated with 1 U of isolated AP at 500 rpm and 37 °C. At predetermined time points (0, 1, 2, 3, 4, 5 and 6 h), 70 μL of samples were withdrawn. In the following, same volume of TNBS solution (0.1% v/v TNBS dissolved in 8% NaHCO3) was added. After incubation of 90 min at 500 rpm and 37 °C, samples of 100 μL were withdrawn and absorbance measurements at 420 nm were performed utilizing a microplate reader. The amount of primary amino groups was determined using a calibration curve having been established with increasing concentrations of L-cysteine HCl. As control, samples was incubated without adding the enzyme.

LogDlipophilic phase/release medium = \log \left( \frac{\text{maximum solubility of zwitterion in the lipophilic phase}}{\text{maximum solubility of zwitterion in release medium}} \right)
of buffer. The plate was incubated at 37 °C while gently shaking at 300 rpm on a shaking board. At predetermined timepoints (0, 1, 2, 3, 4, 5 and 6 h), 100 μL aliquots were taken out from the acceptor chamber and supplanted with the same volume of pre-heated buffer. In the following, 10 μL of 5 M NaOH was added to the withdrawn aliquots and kept for incubation at same conditions for 30 min. The fluorescence intensity of hydrolyzed fluorescein diacetate was analyzed at an excitation wavelength and an emission wavelength of 480 nm and 520 nm, respectively. Cellular uptake was evaluated using Eq. (4):

\[
P_{\text{app}} = \frac{dQ}{dt} \times \frac{1}{A_{C0}}
\]

where \(dQ\) is the flux of permeated nanoemulsion from donor chamber to acceptor chamber during incubation time (nmol), \(dt\) is the time of permeation studies (sec), \(A\) is the mucus gel layer area (cm\(^2\)) and \(C_0\) is the initial fluorescence intensity of nanoemulsion (nmol/cm\(^2\)).

The diffusion ability of the nanoemulsions across the mucus gel layer was further evaluated by the rotating tube method [8]. For this, 300 μL of mucus was filled to a silicon tube (length: 3 cm, diameter: 4 mm) being closed with a silicon cap at one end. Afterward, 50 μL of abovementioned fluorescein diacetate labeled samples (blank formulation FII, p-Tyr loaded formulation FII and p-Tyr loaded formulation FII pretreated with AP) diluted with 10 mL of buffer to each well. Thereafter, 100 μL of 5 M NaOH was added to the well gently shaking at 37 °C for 3 h. The fluorescence intensity of lysate was analyzed at an excitation wavelength and an emission wavelength of 480 nm and 520 nm, respectively. Cellular uptake was evaluated using Eq. (4):

\[
\text{Uptake efficiency} (\%) = \frac{F_t - F_0}{F_{100} - F_0} \times 100
\]

where \(F_t, F_{100}\) and \(F_0\) are the fluorescence intensities of lysate, positive control and negative control, respectively.

2.2.10. Statistical data analysis

Data were analyzed using GraphPad Prism version 5.02. Statistical data analysis between two values were accomplished by the student’s t-test. For multiple comparisons, the two-way analysis of variance (ANOVA) followed by Bonferroni post hoc test was performed. Significance differences were defined as *p ≤ 0.05, **p ≤ 0.01 and ***p ≤ 0.001. All measurements were carried out in triplicate and presented as the mean ± standard deviation (SD).

3. Results and discussion

3.1. Synthesis of phosphorylated tyramine

The required p-Tyr was obtained by reaction of BOC-Tyr with POCl\(_3\) in anhydrous pyridine, followed by hydrolyzation of the ester as illustrated in Fig. 1. The crude product was treated several times with methanol to separate the pyridinium salts. HR-MS, IR and NMR spectroscopy confirmed identity and purity of the target compound.

The [M+H\(^+\)] peak with \(m/z = 218.0552\) (calculated: 218.0577) and its main fragment [M+H\(^+\)−NH\(_2\)] with \(m/z = 201.0289\) (calc. 201.0311) dominate the HR-MS spectrum (Fig. S–1). It is worth mentioning that the spectrum does not include signals referring to the phosphoric diester or triester, which can generally be build up during the reaction course.

The IR spectrum confirms the phosphorylation, too. OH vibrations of phosphoric acid appear to a small extend at 3000–2500 cm\(^{-1}\), while the phosphoric acid ester (P−O−Ar) causes a stretching vibration at 1102 cm\(^{-1}\) (compare Fig. S–2 (BOC-Tyr) and Fig. S–3 (p-Tyr)). The missing NH\(_3\) vibration, the presence of a broad NH\(_2\)\(^\ast\) frequency at about 2900 cm\(^{-1}\) together with the strong P−O− band at 944 cm\(^{-1}\) points to a betaine structure of p-Tyr. Furthermore, IR spectroscopy confirmed the zwitterionic character of p-Tyr. The spectrum exhibited a strong P−O− band at 944 cm\(^{-1}\) and a broad NH\(_2\)\(^\ast\) signal from 3100 cm\(^{-1}\) to 2800 cm\(^{-1}\). In the case of free NH\(_2\) groups symmetric and asymmetric stretching vibrations typically occur as two bands between 3500 cm\(^{-1}\) and 3300 cm\(^{-1}\) of medium intensity.

The \(^1\)H NMR spectrum consists only of the resonances for the tyramine partial structure and documents a successful cleavage of the BOC group (Fig. 3A, see Fig. S–4 for BOC-Tyr). The phosphorylation causes a downfield shift of the H\(_a\) and H\(_b\) protons in BOC-Tyr from \(\delta = 6.72\) and 7.03 to \(\delta = 7.19\) (Hd) and 7.33 (Hc) in p-Tyr (Fig. 3A, Fig. S–4 and Fig. S–5). Additionally, the phosphate ester splits in the \(^31\)P NMR spectrum (Fig. 3B) the resonances of Cp and Cd by coupling to \(^31\)P with \(J = 7.2\) Hz and \(J = 4.4\) Hz.

In the \(^31\)P NMR spectrum, the peak of a monoester typically appears at about −5 ppm (Fig. S–6) and confirmed the structure of p-Tyr (\(\delta = −5.49\)).

The \(^31\)P and \(^1\)H long range correlation experiment excludes the formation of a phosphoramidate, while it proves the phosphorylation of the phenolic hydroxyl moiety. Cross peaks appear only between \(^31\)P and Hd and Hc as well as Hb but not to Ha next to the amino group (Fig. 4). Based on the data discussed above, the formation of a phosphoramidate partial structure, e.g. during the separation process can be excluded [16].
3.2. Development and characterization of nanoemulsions

Five formulations were developed by homogenization of various ratios of oils, surfactants and co-surfactants as mentioned in Table 1 and incorporation of p-Tyr in a concentration of 2% (m/v). The characteristics of excipients utilized for the formation of lipophilic phase were described in Table 2. Nanoemulsions are likely more stable by usage of excipients having low dielectric constant. Higher amount of excipients exhibiting low dielectric constant enhanced the stability of the nanoemulsions on the one hand and stability of incorporated lipophilic complex in the oily droplets on the other hand. The impact of this zwitterion on resulting nanoemulsions was evaluated regarding droplet size and zeta potential. After the incorporation of p-Tyr, formulations appeared as clear (bluish) solutions without any phase separation or precipitation. The zeta potential of oily droplets formed by the three most
promising formulations upon dilution in aqueous media decreased by the incorporation of p-Tyr as shown in Table 3. This decrease in zeta potential confirmed that the phosphate substructures of the zwitterion accumulate on the droplets surface resulting in a negative surface charge. As oils with shorter hydrocarbon chain are likely easier to emulsify compared with oils exhibiting long hydrocarbon chain, Captex 355 and Capmul MCM were used in higher ratio in formulation FII. Accordingly, formulation FII exhibiting a droplet size of 43 nm and a zeta potential of $-8.40 \text{ mV}$ was chosen for further investigations.

Because of their self-emulsifying properties such nanoemulsions can be administered in form of lipophilic preconcentrates emulsifying for instance after oral administration spontaneously in GI-fluids. As these formulations are kinetically stable but in contrast to microemulsions thermodynamically unstable requiring some energy that might be obtained from gastrointestinal mobility for emulsification, we assume that the term nanoemulsion is more appropriate than microemulsion.

3.3. Determination of distribution coefficient ($\log D_{\text{lipophilic phase/release medium}}$)

$\log D_{\text{lipophilic phase/release medium}}$ characterizes the distribution of p-Tyr between oily droplet and the surrounding release medium. The release of the incorporated zwitterion from the nanoemulsion

![Fig. 4. $^{31}$P and $^1$H long range correlation spectroscopy of p-Tyr, recorded in CD$_2$OD/DCl, $^1$H NMR is depicted on the x-axis and $^{31}$P NMR on the y-axis.]
is controlled by a simple diffusion process. As the distance in the oily droplets for this diffusion process is very short, however, equilibrium of the incorporated zwitterion between the oily droplets and the release medium is reached instantaneously [13]. Therefore, the zwitterion needs to remain in the oily droplets until they have reached the epithelium. p-Tyr exhibited a log $D_{lipophilic\ phase/\ release\ medium}$ of 1.86 ± 0.02 indicating a minor amount of zwitterion being released from oily droplets. Additional experiments showed that p-Tyr is practically insoluble in water but to a high extent in the lipophilic phase of the oily droplets.

3.4. Phosphate release studies

Intestinal AP is a membrane-bound metalloenzyme bearing Zn$^{2+}$ and Mg$^{2+}$ ions in its active center being responsible for the hydrolysis of phosphate substructures [17]. Lorenz and Schröder showed that the presence of ethylene diamine tetraacetic acid (EDTA) inhibits phosphate hydrolysis by AP suggesting that Zn$^{2+}$ and Mg$^{2+}$ ions are necessary for the activity of the enzyme [18]. Therefore, ZnCl$_2$ and MgCl$_2$ were applied to assure the activity of isolated AP. Within this study, p-Tyr loaded formulation FII was incubated with isolated AP and phosphate release was evaluated by MLG assay over time. As illustrated in Fig. 5A, a rapid cleavage of phosphate substructures was observed within 3 h of incubation with AP followed by a slower release. In addition, no significant phosphate release was detected in the samples incubated without the enzyme. This result demonstrated that the cleavage of the phosphate moieties from the droplet surface is completely enzyme-dependent. As AP rapidly cleaves phosphate groups from tyrosine subunits on large and even hydrophobic proteins in an efficient manner, cleavage of phosphate on the smooth surface of these oily droplets likely does not represent a greater challenge for this enzyme. Results obtained are in good agreement with a previous study in which a rapid phosphate cleavage from the droplets surface was observed within 60 min of incubation followed by a slower cleavage behavior [4].

In order to investigate the phosphate release behavior on the epithelium, human colonic adenocarcinoma cells were chosen as in vitro cell model. Phosphate cleavage from surface of oily droplets depends on cleavage by AP expressed by these cells. Wu et al. verified that Caco-2 cell lines express AP in higher concentrations than HT-29-MTX-E12 cell lines besides having the morphological and biochemical features similar to intestinal brush-border membranes [5]. Jumarie et al. described that Caco-2 cell lines can modulate brush-border membrane enzyme activities during cell proliferation and differentiation [19]. Therefore, Caco-2 cell line was chosen to measure the cleaved phosphate by AP over time. As demonstrated in Fig. 5B, a rapid phosphate release from p-Tyr loaded oily droplets was observed within 3 h of incubation followed by a slow release. This behavior might be described by the inhibitory influence of free phosphate. Bound phosphate ions can be considered as a stimulus for AP activity. In comparison, free phosphate ions are deliberated as competitive inhibitors for AP resulting in a lower activity. This release behavior of phosphate substructures was in agreement with the release of phosphate by isolated AP. The phosphate cleavage from the oily droplets was also estimated by adding phosphatase inhibitor cocktail II in order to inhibit the activity of AP. However, a trivial amount of phosphate was cleaved as this inhibitor could likely not completely obstruct the activity of the enzyme. To some extent also other unspecific membrane bound enzymes might be involved in this cleavage process that are not or just partially inhibited by this cocktail. As Caco-2 cells exhibit just half of the activity of AP than human intestinal mucosa [20], a comparatively lower amount of phosphate cleavage was observed. Other mucosal tissues such as pulmonary mucosa exhibit an even higher enzymatic activity of AP [21] then the intestinal mucosa. Furthermore, inorganic phosphate is known as a competitive inhibitor of AP. Thus the released phosphate might in turn inhibit the enzyme and slow down its enzymatic activity. According to these considerations the activity of AP will likely be much higher in vivo. Whether this enzymatic activity will be higher enough to guarantee a pronounced change in zeta potential in vivo, however, will need to be tested in follow up studies.

3.5. Determination of zeta potential change

Results of time-dependent phosphate release studies in the presence of AP were confirmed by the zeta potential changing behavior of the oily droplets. As illustrated in Fig. 6, a significant zeta potential change from −8.4 mV to +1.3 mV was observed within 6 h. In parallel, no significant zeta potential change was detected when AP was omitted. In general, a rapid rise in zeta potential was detected within the first 3 h followed by a slower rise thereafter. This change in zeta potential over time was in good agreement with the phosphate release from the oily droplets shown in Fig. 5A and Fig. 5B. It provided evidence for the accumulation of p-Tyr on the interphase of the nanoemulsion, as otherwise the zeta potential cannot change. If the zwitterions are on the one hand embedded inside in the oily droplets, they will not be cleaved by AP. If the zwitterions are on the other hand released in the aqueous phase their cleavage by AP would not have an impact on the zeta potential of the oily droplets.
3.6. Amino group determination

After enzymatic cleavage of the phosphate moiety the amino group flipped out of the lipophilic phase and accumulated on the surface of the oily droplets resulting in a shift in zeta potential. The amount of primary amino groups was quantified by TNBS assay after cleavage of phosphate moieties during incubation of p-Tyr loaded nanoemulsion with isolated AP as shown in Fig. 7. The amount of the amino groups on the droplets surface was significantly augmented over time. Nazir et al. reported that the amount of the primary amino group on the surface of phosphorylated serine-oleylamine loaded oily droplets raised significantly during incubation with AP over time [4]. A similar migration of amino groups to the surface of the oily droplets after phosphate cleavage was observed in another study utilizing phosphorylated 12-amino-dodecanol as a zeta potential changing agent [15]. These results provided additional evidence for the accumulation of p-Tyr on the interphase of the nanoemulsion, as an increase in primary amino groups in the aqueous phase due to the addition of AP can only be explained by a phosphate cleavage of p-Tyr on the droplet surface followed by a change in orientation of the former zwitterion with its amino group heading now towards the aqueous phase.

3.7. Impact on viability of Caco-2 cells

Resazurin assay was accomplished to evaluate the in-vitro cytotoxic behavior of p-Tyr loaded nanoemulsion on Caco-2 cells. This assay is based on the metabolic activity of the cells as viable cells have the capability to catalyze non-fluorescent oxidized resazurin (blue) to high-fluorescence reduced resorufin (pink) [22]. As illustrated in Fig. 8, p-Tyr solution in HBS showed a minor cytotoxic effect on Caco-2 cells at a final concentration of 0.25%. Nanoemulsion FII containing p-Tyr having been diluted 1:500 and 1:1000 in HBS pH 7.4 showed also a cell viability above 80%. In contrast, cationic surfactants such as cetrimonium bromide, benzalkonium chloride and cetylpolyridinum chloride loaded into oily droplets to induce a positive zeta potential exhibited high cytotoxic effects. Suchaoin et al., for instance, observed that the incorporation of cationic surfactants such as cetrimonium bromide and cetylpyridinium chloride in a concentration of 0.002% in oily droplets caused a drop in cell viability below 20% [8]. Similarly, Lam et al. showed that cationic surfactants such as benzalkonium chloride, alkyltrimethylammonium bromide and hexadecylpyridinium chloride display a cytotoxic effect on Caco-2 cells even at a concentration of 0.002%. The incorporation of these cationic surfactants in oily droplets enhanced even their cytotoxic potential [23].

3.8. Mucus permeation studies

Based on the similarity between human intestinal and porcine intestinal mucins in structure and molecular mass, porcine intestinal mucus was utilized as a mucus model [5,24]. In order to evaluate the permeation of p-Tyr loaded oily droplets through the mucus gel layer, a Transwell® insert method was utilized. The p-Tyr loaded nanoemulsion was incubated with AP for 3 h prior to the experiment in order to cleave phosphate moieties. As shown in Fig. 9A, p-Tyr loaded oily droplets permeated the mucus gel layer to a 8.59-fold higher extent than AP pretreated p-Tyr loaded oily droplets. These results can be elucidated by the electrostatic repulsion between negatively charged p-Tyr loaded droplets and...
negative charges of sulfonic and sialic acid moieties found in mucus facilitating the movement of droplets through the mucus. A similar behavior was observed in a previous study by Suchaoin et al., where a negatively charged phosphorylated nanoemulsion was shown to move more rapidly across the mucus layer than a nanoemulsion pretreated with AP [8].

$P_{app}$ values of blank droplets and p-Tyr loaded droplets with and without AP pretreatment are demonstrated in Fig. 9B. p-Tyr loaded droplets showed the highest $P_{app}$ values compared to blank droplets and AP pretreated droplets. These results indicated that phosphate moieties on the surface of the droplets prevent the entrapment and aggregation of droplets in the negatively charged mucus gel layer resulting in a rapid mucus permeation.

The diffusion rate of p-Tyr loaded nanoemulsion into the depth of the mucus layer was further evaluated by rotating silicon tube method. The results are illustrated in Fig. 9C. p-Tyr loaded droplets diffused to a 2.5-fold higher extent in segments 1 compared to blank droplets, whereas the zwitterion loaded oily droplets diffused to a 5.83-fold higher extent in segment 1 compared to p-Tyr loaded droplets pre-treated with isolated AP. The results are in accordance with Transwell permeation studies confirming the electrostatic repulsion between negatively charged mucus and negatively charged p-Tyr loaded droplets resulting in rapid diffusion across the mucus gel layer.

3.9. In-vitro cellular uptake

After permeation across the mucus gel layer, the absorption barrier of epithelium per se represents another confronting barrier [25]. Overcoming this barrier requires hydrophobic and positively charged surface properties of nanocarriers [26,27]. The cleavage of the negatively charged phosphate groups and the subsequent accumulation of positively charged amino groups on the droplets due to membrane-bound AP has a great impact on their uptake by epithelial cells. These amino groups can intensively interact with anionic heparin sulfate proteoglycans on the surface of epithelial cells depolarizing their membrane and facilitating cellular uptake [28]. Caco-2 cell line was utilized to measure the magnitude of cellular uptake of p-Tyr loaded droplets formed by formulation FII as this cell line has structural and morphological

![Image](https://example.com/image.png)
resemblance with enterocytes and the foremost category of the cells available in the small intestine are enterocytes [29]. The uptake of p-Tyr loaded droplets was assessed on Caco-2 cells. As control, samples were also incubated on Caco-2 cells with the addition of phosphatase inhibitor cocktail II. The cellular uptake of p-Tyr loaded droplets increased 2.4-fold compared to cells whose phosphatase activity was inhibited as illustrated in Fig. 10. These results confirmed that phosphate cleavage on the surface of the nanoemulsions is responsible for the change in zeta potential resulting in enhanced cellular uptake. Wu et al. described that poly(lactic-co-glycolic acid) nanoparticles comprising phosphoserine and octa-arginine exhibited 72% less cellular uptake in presence of phosphatase inhibitors [5]. Similarly, Nazir et al. observed a 2-fold higher cellular uptake of oily droplets containing phosphorylated serine-oleylamine as zeta potential changing agent in absence of phosphatase inhibitor [4].

4. Conclusion

Up to date, all zeta potential changing nanoemulsions contain a cationic surfactant after phosphate cleavage being responsible for a change to a positive zeta potential [8]. These resulting lipophilic cationic surfactants seems to be problematic from the toxicological point of view. Within the present study, this shortcoming was addressed by investigating the zeta potential changing potential employing an alternative to cationic surfactants. We synthesized p-Tyr, a simple zwitterion, exhibiting a phosphate substructure and an amino substructure on a simple small molecule and incorporated it into nanoemulsions. These negatively charged p-Tyr loaded oily droplets permeate more rapidly across the mucus gel layer due to electrostatic repulsion from the negative charge of the mucus [30]. After permeation through the mucus, this zwitterion changes the zeta potential of the oily droplets due to cleavage of the phosphate substructure by membrane-bound AP resulting in an accumulation of its amino substructure on the droplet surface. This change in zeta potential improved the uptake of oily droplets by epithelial cells 2.4-fold whereas previously reported zeta potential changing self-emulsifying drug delivery systems exhibited a cellular uptake improvement of just up to 2-fold [4]. Hence, zwitterions could be identified as promising auxiliary agents for the development of zeta potential changing nanoemulsions in order to overcome the diffusion barrier of the mucus and absorption barrier of the epithelium. Based on the knowledge gained from this first proof of concept study further likely even more efficient zwitterions can be discovered within the next years promoting the potential of this new technology.

CRediT authorship contribution statement

Faezeh Sharifi: Investigation, Methodology, Validation, Visualization, Writing - original draft. Mansour Jahangiri: Visualization. Imran Nazir: Investigation, Methodology, Validation, Writing - original draft, Writing - review & editing. Mulazim Hussain Asim: Investigation, Visualization, Writing - review & editing. Pedram Ebrahimnejad: Writing - review & editing. Andrea Hupfau: Investigation, Writing - review & editing. Ronald Gust: Formal analysis, Writing - review & editing. Andreas Bernkop-Schnürch: Conceptualization, Resources, Funding acquisition, Supervision, Project administration.

Declaration of Competing Interest

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.

Acknowledgment

We are highly thankful for the support from the Ministry of Science, Research and Technology of Iran, the Austrian Agency for International Cooperation in Education and Research (ÖAD), Austria and Higher Education Commission (HEC), Pakistan. The Austrian Research Promotion Agency FFG (West Austrian BioNMR 858017) is kindly acknowledged. The research leading to these results has received funding from the Austrian Science Fund (FWF), Austria under the project number ZFP0268-B30.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2020.11.054.

References

[1] S. Bonengel, F. Prüfert, G. Perera, J. Schauer, A. Bernkop-Schnürch, Polyethylene imine-6-phosphogluconic acid nanoparticles—a novel zeta potential changing system, Int. J. Pharm. 483 (1–2) (2015) 19–25.
[2] I. Nazir, C. Leischner, B. Le-Vinh, A. Bernkop-Schnürch, Surface phosphorylation of nanoparticles by hexokinase: a powerful tool for cellular uptake improvement, J. Colloid Interface Sci. 516 (2018) 384–391.
[3] B. Le-Vinh, N.-M.N. Le, I. Nazir, B. Matuszczak, A. Bernkop-Schnürch, Chitosan based mucoadhesive zeta potential changing nanoparticles for effective mucosal drug delivery, Int. J. Biol. Macromol. 133 (2019) 647–655.
[4] I. Nazir, A. Fürst, N. Lupo, A. Hupfau, R. Gust, A. Bernkop-Schnürch, Zeta potential changing self-emulsifying drug delivery systems: a promising strategy to sequentially overcome mucus and epithelial barrier, Eur. J. Pharm. Biopharm. 144 (2019) 40–49.
[5] J. Wu, Y. Zheng, M. Liu, W. Shan, Z. Zhang, Y. Huang, Biomimetic virusslike and charge reversible nanostructures to sequentially overcome mucus and epithelial barriers for oral insulin delivery, ACS Appl. Mater. Interfaces 10 (12) (2018) 9916–9928.
[6] C. Dumont, S. Bourgeois, H. Fessi, V. Jannin, Lipid-based nanosuspensions for oral delivery of peptides, a critical review, Int. J. Pharm. 541 (2021) 2018–115.
[7] K. AboulFotouh, A.A. Allam, M. El-Badry, A.M. El-Sayed, Role of self-emulsifying drug delivery systems in optimizing the oral delivery of hydrophilic macromolecules and reducing interindividual variability, Colloids Surf. B Biointerfaces 167 (2018) 82–92.
[8] W. Suchaoin, I.P. de Sousa, K. Netsomboon, H.T. Lam, F. Laffleur, A. Bernkop-Schnürch, Development and in vitro evaluation of zeta potential changing self-emulsifying drug delivery systems for enhanced mucus permeation, Int. J. Pharm. 510 (1) (2016) 255–262.
[9] G. Andersen, P. Marcinek, N. Sulzinger, P. Schieberle, D. Krautwurst, Food sources and biomolecular targets of tyramine, Nutr. Rev. 77 (2) (2019) 107–115.
[10] G. Jairath, P.K. Singh, R.S. Darbur, M. Rani, M. Chaudhari, Biogenic amines in meat and meat products and its public health significance: a review, J. Food Sci. Technol. 52 (11) (2015) 6835–6846.
[11] M.S. Benedetti, T. Boucher, A. Carlsson, C.J. Fowler, Intestinal metabolism of tyramine by both forms of monoamine oxidase in the rat, Biochem. Pharmacol. 32 (1) (1983) 47–52.
[12] J. Zhao, S. Wang, S. Lu, X. Bao, J. Sun, X. Yang, An enzyme cascade-triggered fluorescent and chromogenic reaction applied in enzyme activity assay and immunoassay, Anal. Chem. 90 (12) (2018) 7754–7760.
[13] A. Bernkop-Schnürch, A. Jalil, Do drug release studies from SEDDS make any sense?, J. Control. Release 271 (2018) 55–59.
[14] J. Feng, Y. Chen, J. Pu, X. Yang, C. Zhang, S. Zhu, et al., An improved malachite green assay of phosphate: mechanism and application, Anal. Biochem. 409 (1) (2011) 144–149.
[15] F. Sharifi, I. Nazir, M.H. Asim, M. Jahangiri, P. Ebrahimnejad, B. Matuszczak, et al., Zeta potential changing self-emulsifying drug delivery systems utilizing a novel Janus-headed surfactant: a promising strategy for enhanced mucus permeation, J. Mol. Liq. 291 (2019) 111283.
[16] K. Ghoshvand, S. Ghadimi, H. Naderimanesh, A. Forouzanfar, Dependence of the long-range phosphorus–hydrogen coupling constant nJP–H (n = 3, 6, 7) on the bond order between phosphorus and its substituents: preparation and spectroscopic characterization of several phosphoromadiges, Magn. Reson. Chem. 39 (11) (2001) 684–688.
[17] K.M. Holz, B. Stec, E.R. Kantrowitz, A model of the transition state in the alkaline phosphatase reaction, J. Biol. Chem. 274 (13) (1999) 8351–8354.
[18] B. Lorenz, H.C. Schröder, Mammalian intestinal alkaline phosphatase acts as highly active exopolysphatase, Biochim. et Biophys. Acta (BBA)-Protein Struct. Mol. Enzymol. 1547 (2) (2001) 254–261.
[19] C. Jumarie, C. Malo, Alkaline phosphatase and peptidase activities in Caco-2 cells: differential response to triiodothyronine, Vetri Cell. Dev. Biol.-Ani. 30 (11) (1994) 753–760.
[20] M. Pinto, Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture, Biol. Cell. 47 (1983) 323–330.

[21] F. Becq, T.J. Jensen, X.B. Chang, A. Savoia, J.M. Rommens, L.C. Tsui, et al., Phosphatase inhibitors activate normal and defective CFTR chloride channels, Proc. Natl. Acad. Sci. U S A. 91 (19) (1994) 9160–9164.

[22] H.-X. Zhang, G.-H. Du, J.-T. Zhang, Assay of mitochondrial functions by resazurin in vitro, Acta Pharmacol. Sin. 25 (3) (2004) 385–389.

[23] H.T. Lam, B. Le-Vinh, T.N.Q. Phan, A. Bernkop-Schnürch, Self-emulsifying drug delivery systems and cationic surfactants: do they potentiate each other in cytotoxicity?, J. Pharm. Pharmacol. 71 (2) (2019) 156–166.

[24] A.-C. Groo, F. Lagarce, Mucus models to evaluate nanomedicines for diffusion, Drug Discovery Today 19 (8) (2014) 1097–1108.

[25] I. Pepić, J. Lovrić, J. Filipović-Grčić, How do polymeric micelles cross epithelial barriers?, Eur. J. Pharm. Sci. 50 (1) (2013) 42–55.

[26] S. Alqahtani, L. Simon, C.E. Astete, A. Alayoubi, P.W. Sylvester, S. Nazzal, et al., Cellular uptake, antioxidant and antiproliferative activity of entrapped α-tocopherol and γ-tocotrienol in poly (lactic-co-glycolic) acid (PLGA) and chitosan covered PLGA nanoparticles (PLGA-Chi), J. Colloid Interface Sci. 445 (2015) 243–251.

[27] Y. Wang, Y. Cui, Y. Zhao, Q. Zhao, B. He, Q. Zhang, et al., Effects of surface modification and size on oral drug delivery of mesoporous silica formulation, J. Colloid Interface Sci. 513 (2018) 736–747.

[28] A. Komin, L. Russell, K.A. Hristova, P. Searson, Peptide-based strategies for enhanced cell uptake, transcellular transport, and circulation: mechanisms and challenges, Adv. Drug Deliv. Rev. 110 (2017) 52–64.

[29] A.L. Kauffman, A.V. Gyurdieva, J.R. Mabus, C. Ferguson, Z. Yan, P.J. Hornby, Alternative functional in vitro models of human intestinal epithelia, Front. Pharmacol. 4 (2013) 79.

[30] L. Wu, W. Shan, Z. Zhang, Y. Huang, Engineering nanomaterials to overcome the mucosal barrier by modulating surface properties, Adv. Drug Deliv. Rev. 124 (2018) 150–163.