Zeta potential changing nanoemulsions based on phosphate moiety cleavage of a PEGylated surfactant

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The aim of this study was to develop and evaluate a zeta potential changing nanoemulsion (NE) containing polyoxyethylene (9) nonylphenol monophosphate ester (PNPP) as emulsifier. 2% (v/v) of PNPP and 0.18% (5 μM) of cetyltrimethylammonium bromide (CTAB) were incorporated into the lipophilic NE preconcentrate (PEG-40 castor oil, glyceryl tricaprylate/tricaprate, propylene glycol dicaprylocaprate, propylene glycol monolaurate, highly purified diethyleneglycol glycol monoethyl ether; 20/20/10/30/20, v/v). After diluting the lipophilic preconcentrate, resulting NE was analyzed regarding droplet size, polydispersity index (PDI), storage stability and zeta potential change after incubation with intestinal alkaline phosphatase (IAP). Phosphate release due to cleavage by IAP was quantified by malachite green assay and toxicity as well as cellular uptake behavior on Caco-2 cells was determined. The NE containing PNPP and CTAB displayed a droplet size of 113 nm and a PDI of 0.20. It was stable over 4 h. A zeta potential change from −33.7 to +8.2 mV was observed due to cleavage of phosphate groups by isolated IAP. PNPP could be identified as non-toxic up to concentrations of 0.01% (v/v). Furthermore, an enhanced cellular uptake of the NE changing its zeta potential on Caco-2 cells was determined.

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1. Introduction

Nanoemulsions (NE) are isotropic dispersions of two non-miscible liquids with droplet sizes in the nanometric range. In case of o/w NE an oily phase is dispersed in an aqueous medium and stabilized by appropriate surfactants. They can be prepared by “high-energy” techniques such as ultrasonication and high-pressure homogenization or alternatively be designed as self-emulsifying systems. As the oily droplets showed high potential as nanocarriers for oral drug delivery, the pharmaceutical industry discovered NE as useful formulation tool [1–3]. However, upon oral administration NE have to overcome several barriers before facilitating systemic absorption of encapsulated drugs [4]. The efficiency of NE to take these hurdles strongly depends on their zeta potential. To achieve enhanced mucus permeation properties a negative zeta potential is favored [5,6], as the mucus contains large amounts of mucins with anionic sialic- and sulfonic acid moieties immobilizing positively charged carriers due to electrostatic attraction [7,8]. After crossing the mucus barrier, a positive zeta potential is preferred to enhance cellular uptake via ionic attraction of these nanocarriers with the negatively charged cell surface [9–11]. Consequently, anionic nanocarriers with the ability to shift their zeta potential to positive once having reached the absorption membrane hold great promise as delivery systems.

Due to the incorporation of phosphorylated surfactants in NE a zeta potential change from negative to positive values can be achieved via cleavage of the phosphate moieties by the membrane bound enzyme intestinal alkaline phosphatase (IAP) [12–15]. For an efficient cleavage by IAP, however, the accessibility of the phosphate moieties on the surface of the oily droplets is crucial. As adequate emulsification properties and stability of NE in general require the addition of strong PEGylated emulsifiers, the formation of a PEG corona around the lipophilic core is evident. When the phosphate moiety is bound directly to the hydrophobic tail of the surfactant anchoring it in the oily core of the droplets, its movement at the oil-PEG interface and a consequently limited access for IAP due to the shielding effect of the PEG corona is

Abbreviations: CPC, cetylpyridinium chloride; CTAB, cetyltrimethylammonium bromide; DTAB, n-dodecyltrimethylammonium bromide; EQ, esterquat (bis-(isostearoyl/oleoyl isopropyl) dimonium methosulfate); HBS, HEPES buffered saline; IAP, intestinal alkaline phosphatase; LPNE, lipophilic phase of nanoemulsion; MEM, Eagles minimum essential medium; MLG, malachite green; NE, nanoemulsion; OM, OptiMEM; PDI, polydispersity index; PIC, phosphatase inhibitor cocktail; PNPP, polyoxyethylene (9) nonylphenol monophosphate ester; POAP, N,N′-bis(polyoxyethylene) oleylamine bisphosphate.

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likely. The resulting zeta potential change is minor. In order to overcome this PEG corona, a hydrophilic linker between the phosphate group and the lipophilic tail of the surfactant seems useful. Wolf et al. synthesized such a surfactant, namely \( \text{N}^\text{N}^\text{N}^\text{bis(polyoxyethylene)} \) oleylamine bisphosphate (POAP) and achieved a zeta potential shift of \( \Delta 21.6 \text{ mV} \) underlining the potential of such surfactants [16].

Although a proof-of-concept could be provided for this type of phosphorylated surfactant, its tertiary amine substructure turned out to be too toxic for further developments. Combining the required cationic moieties with the phosphate substructure on the same molecule was obviously a too ambitious concept. Furthermore, two phosphorylated PEG chains on the same surfactant might hinder the accessibility of IAP as well.

The purpose of this study was therefore to develop a zeta potential changing system based on a different phosphorylated surfactant exhibiting a PEG spacer. To achieve this goal, polyoxyethylene (9) nonylphenol monophosphate ester (PNPP), as illustrated in Fig. 1, was incorporated in a NE. The resulting formulation should demonstrate a reduced cytotoxicity compared to previous systems including substances such as POAP due to the lack of a positive charged molecule moiety. Furthermore, the single-chain hydrophilic PEG linker of PNPP might enable a more efficient phosphate cleavage by IAP leading to a higher zeta potential change in comparison to already established systems.

2. Materials and methods

2.1. Materials

PEG-40 castor oil (Kolliphor® RH40) was a gift from BASF SE, Germany. Glycerol tricaprylate/tricaprate (Captex® 300) was donated from Abitec Corporation, USA. Gattefossé, France provided propylene glycol dicaprylocaprate (Labrafil™ PG), propylene glycol monolaurate (Lauroglycol™ 90) and highly purified diethyleneglycol monoethyl ether (Transcutol® HP). Ashland Inc., Switzerland, supplied Polyoxylethylene (9) nonylphenol monophosphate ester (PNPP; Dextrol™ OC-20). The esterquat bis-(iso/stearyol/oleoyl isopropyl) dimonium methosulfate (EQ; VARIOSOFT™ EQ 100) was obtained from Evonik Industries AG, Germany. Alkaline phosphatase from calf intestine with an activity of approximately 3000 U·mg\(^{-1}\) protein, whereby 1 unit catalyzes the hydrolysis of 1 \( \mu \text{mol of 4-nitrophenyl phosphate per minute at 37 } ^\circ\text{C} \) in DEA buffer pH 9.8, was purchased from SERVA Electrophoresis GmbH, Germany. Sigma-Aldrich, Austria was the supplier of phosphatase inhibitor cocktail 2. Lumogen® F yellow 083 was purchased from BASF SE, Germany. All other reagents, solvents and chemicals were of analytical grade and received from commercial sources.

2.2. Purification of PNPP

In order to remove residues of phosphoric acid contaminating PNPP flash column chromatography (silica gel 60, 400–220 mesh, Carl Roth, Germany) was conducted. Therefore, a solvent mixture consisting of isopropanol, 25% ammonia solution and water was utilized (7/2/1, v/v/v) and subsequently evaporated under vacuum. Thereafter, precipitated silica was removed via centrifugation giving PNPP as a viscous yellowish liquid with a yield of 76%. All further experiments were performed with this purified PNPP.

2.3. Preparation and characterization of nanoemulsions

2.3.1. Preparation of nanoemulsions

The lipophilic phase of NE without PNPP (LPNE\(_{\text{Blank}}\)) was prepared by homogenizing components as listed in Table 1. For LPNE containing PNPP (LPNE\(_{\text{PNPP}}\)), 2% (v/v) of PEG-40 castor oil were replaced with PNPP. Furthermore, the cationic surfactants cetylpyridinium chloride (CPC), cetyltrimethylammonium bromide (CTAB), n-dodecyltrimethylammonium bromide (DTAB) as well as the esterquat bis-(iso/stearyol/oleoyl isopropyl) dimonium methosulfate (EQ) were incorporated in a concentration of 5 \( \mu \text{M} \) into LPNE\(_{\text{PNPP}}\) by vortexing and treatment with ultrasound under homogenous a phase was obtained. As CTAB turned out to be the most promising cationic surfactant, it was also tested in concentrations of 1 \( \mu \text{M}, 15 \mu \text{M}, 20 \mu \text{M} \) as well as 30 \( \mu \text{M} \). As all formulations displayed self-emulsifying properties, emulsions were prepared by diluting the lipophilic phase 1:100 (v/v) with demineralized water and shaking at 300 rpm and 37 °C for 1 min. NE were incubated at 37 °C under shaking at 300 rpm and characterized regarding droplet size after 0 as well as 4 h (Nanobrook 90P plus PALS, Brookhaven Instruments, USA).

2.3.2. Zeta potential change analysis

Zeta potential change was induced by the addition of 5 \( \mu \text{L}-\text{ml}^{-1} \) of intestinal alkaline phosphate resulting in an enzymatic activity of 5 U·ml\(^{-1}\) to LPNE having been diluted 1:100 (v/v) with demineralized water. Thereafter, zeta potential of samples shaken at 300 rpm and 37 °C was analyzed at 0 and 4 h. The particle analyzer analyzer analyzing a voltage of 4 V and a field frequency of 2 Hz. Emulsions of LPNE\(_{\text{Blank}}\) serving as reference were prepared and investigated as described above. LPNE containing 5 \( \mu \text{M} \) of CTAB was additionally investigated at 1, 2 and 3 h.

2.4. Phosphate release studies

For phosphate release studies, a malachite green (MLG) assay was conducted based on an already described method [16,17]. In brief, 1.25 mL of an aqueous ammonium molybdate tetrahydrate solution (7.5%, w/v), 0.1 mL of Tween 20 (11%, v/v) in water as well as 5 mL of a malachite green oxalate solution (0.12%, w/v) prepared with 2.25 M sulfuric acid were combined in order to obtain the MLG reagent. Afterwards, the amount of inorganic phosphate within samples was determined by mixing 20 \( \mu \text{L} \) of 1 M sulfuric acid, 180 \( \mu \text{L} \) of test sample as well as 50 \( \mu \text{L} \) of the MLG reagent in a 96-well plate and subsequent measurement of the absorbance at 620 nm after shaking at 150 rpm for 10 min. For calculating inorganic phosphate concentrations, a calibration curve \((R^2 = 0.997)\) was prepared within a concentration range of 0.5 to 50 \( \mu \text{M} \) using an emulsion prepared with LPNE\(_{\text{blank}}\) as dilution medium to address the influence of surfactants on the analysis [18].

| Components | LPNE Without PNPP | With PNPP |
|------------|-------------------|----------|
| PEG-40 castor oil (Kolliphor® RH 40) | 20 | 18 |
| Glycerol tricaprylate/tricaprate (Captex® 300) | 20 | 20 |
| Propylene glycol dicaprylocaprate (Labrafil™ PG) | 10 | 10 |
| Propylene glycol monolaurate (Lauroglycol™ 90) | 30 | 30 |
| Highly purified diethyleneglycol monoethyl ether (Transcutol® HP) | 20 | 20 |
| Polyoxylethylene (9) nonylphenol monophosphate ester (PNPP; Dextrol™ OC-20) | – | 2 |
2.4.1. Phosphate release studies catalyzed by isolated phosphatase
As LPNE\textsubscript{PNNP} containing 5 μM of CTAB (LPNE\textsubscript{PNNP+CTAB}) turned out to be the most promising formulation after zeta potential analysis, the released phosphate after enzyme addition was investigated. Therefore, 5 μL·mL\textsuperscript{−1} of IAP with an enzymatic activity of 0.5 U·mL\textsuperscript{−1} were added to LPNE\textsubscript{PNNP+CTAB} having been diluted 1:1000 (v/v) with demineralized water. While incubating the NE at 37 °C and 300 rpm, aliquots of 180 μL were withdrawn at 0, 1, 2, 3 as well as 4 h and analyzed via MLG assay. LPNE\textsubscript{Blank}, having been treated in the same way as LPNE\textsubscript{PNNP} served as reference.

2.4.2. Caco-2 cells induced phosphate release
Phosphate release of LPNE\textsubscript{Blank} as well as LPNE\textsubscript{PNNP+CTAB} was investigated on Caco-2 cells as they represent an intestinal cell line expressing IAP [19,20]. Cells were cultured in Eagles minimal essential medium (MEM) supplemented with 10% fetal bovine serum as well as 1% penicillin-streptomycin after seeding them in 24-well plates with a density of 2 × 10\textsuperscript{3} per well. Cells were incubated at 90% relative humidity, 5% CO\textsubscript{2} as well as 37 °C and MEM was changed every 48 h. After 14 days cell monolayers were washed trice with HEPES buffered saline pH 7.4 (HBS, 20 mM HEPES, 5.5 mM glucose, 137 mM NaCl, 5 mM KCl, 1 mM CaCl\textsubscript{2}) and treated with 500 μL of HBS containing 1% (v/v) Phosphate Inhibitor Cocktail 2 (PIC) or 500 μL of pure HBS for 30 min. Then, 1.1 mL of LPNE\textsubscript{PNNP+CTAB} diluted 1:1000 (v/v) with HBS or with HBS containing 1% (v/v) PIC were transferred into each well, whereby HBS and with 1% (v/v) PIC served as references. While incubating cells at 37 °C, aliquots of 180 μL were taken at predetermined time points and immediately mixed with 20 μL of 1 M sulfuric acid to inhibit any further enzymatic activity. Afterwards, the amount of released phosphate was determined by MLG assay.

2.5. Cytotoxicity studies on Caco-2 cells
In order to investigate the impact of NE prepared from LPNE\textsubscript{Blank} as well as LPNE\textsubscript{PNNP+CTAB} or plain PNPP on viability of Caco-2 cells, a resazurin assay was conducted [21]. Cells having been cultured in 24-well plates as described above were washed twice with HBS. 500 μL of LPNE\textsubscript{Blank} and LPNE\textsubscript{PNNP+CTAB} both having been diluted 1:1000 (v/v) with HBS or PNPP dissolved in HBS in a concentration range from 0.002% to 0.02% (v/v) were transferred into the wells. As positive control HBS and as negative control 2% (v/v) Triton X100 in HBS were used. As positive control 0.1% (w/w) Lumogen® F yellow 083 and diluted 1:10,000 (v/v) with OM or with OM containing 1% (v/v) PIC. Subsequently, cells were washed with preheated OM thrice and nuclei were stained utilizing Hoechst 33528 (1 μg·mL\textsuperscript{−1}). Equal confocal settings were applied for recording all fluorescence images and image post-processing was conducted by ImageJ whereby the yz- and xz-projections were obtained from 5 xy-images of an image stack taken at 0.2 μm z-step length. Furthermore, spectral unmixing was used in order to erase fluorescence bleed-through between detection channels and 2D image filtering was applied via a Gaussian filter.

2.7. Statistical data analysis
Results are presented as means ± SD. One-way ANOVA followed by Tukey post hoc test with a level of significance of 0.05 was utilized for statistical data evaluation (IBM SPSS Statistics, version 22).

3. Results and discussion

3.1. Preparation and characterization of NE

3.1.1. Evaluation without cationic surfactants
As depicted in Table 2, NE displayed stable droplet sizes over 4 h and polydispersity indexes (PDIs) ≤ 0.2 indicating monodisperse systems. For NE with PNPP (NE\textsubscript{PNPP}), however, a larger droplet size of about 40 nm compared to NE without PNPP (NE\textsubscript{Blank}) was found. This increase might be explained by a different emulsification behavior of LPNE\textsubscript{PNNP} compared to LPNE\textsubscript{Blank}, as 2% (v/v) of the surfactant PEG–40 castor oil were replaced with PNPP. Regard to zeta potential profiles of both formulations upon incubation with IAP, a strong increase of Δ33.4 mV was observed for NE\textsubscript{PNPP} whereas NE\textsubscript{Blank} showed no significant change within 4 h (Table 2). The measured shift amplitude was even more than 1.5-fold higher compared to the so far most pronounced change in zeta potential reported in the literature [16] demonstrating the potential of PNPP.

3.1.2. Evaluation of different cationic surfactants
Despite this change in zeta potential, the formulation NE\textsubscript{PNPP} still exhibited a slightly negative potential of −6.7 mV after 4 h. Thus, in order to reach a positive zeta potential, different cationic surfactants were incorporated in LPNE\textsubscript{PNNP} in a concentration of 5 μM for comparison reasons. In case of all surfactants, a minor decrease in droplet size in a range of Δ10 to 20 nm in relation to NE\textsubscript{PNPP} was analyzed, as shown in Table 3. Furthermore, droplet size remained stable for 4 h and PDIs were below 0.2 for all tested formulations. Accordingly, the emulsification characteristics of NE were altered only to a minor extent by incorporating various cationic surfactants.

Comparing the IAP-mediated zeta potential change, no statistical difference between CPC, CTAB and DTAB could be found, whereas this change was less pronounced in case of EQ. As the relative highest shift in zeta potential with Δ41.9 mV and the significant (p ≤ 0.05) most positive zeta potential of +8.2 mV was achieved with CTAB, it was used for more detailed studies.

| Formulation | Droplet size (nm) 0 h | Droplet size (nm) 4 h | PDI 0 h | PDI 4 h |
|-------------|----------------------|----------------------|--------|--------|
| NE\textsubscript{Blank} | 84 ± 2 | 82 ± 1 | 0.16 ± 0.01 | 0.16 ± 0.01 |
| NE\textsubscript{PNPP} | 125 ± 7 | 120 ± 6 | 0.17 ± 0.01 | 0.16 ± 0.02 |

| Formulation | Zeta potential (mV) 0 h | Zeta potential (mV) 4 h | Zeta potential increase (ΔmV) in 4 h |
|-------------|------------------------|------------------------|-------------------------------------|
| NE\textsubscript{Blank} | −6.8 ± 0.2 | −7.0 ± 0.9 | −0.2 ± 0.7 |
| NE\textsubscript{PNPP} | −40.1 ± 1.0 | −6.7 ± 1.1 | 33.4 ± 0.6 |
3.1.3. Evaluation of different concentrations of cationic surfactant

CTAB was incorporated into LPNEPNPP in various concentrations to investigate the influence on droplet size and zeta potential change. With increasing concentrations of CTAB droplet size decreased likely caused by a concentration dependent influence of the cationic surfactant on the emulsification characteristics of LPNEPNPP. Correspondingly, the shift in zeta potential was attenuated, as for 1 μM CTAB the zeta potential increased by Δ46.5 mV, whereas a concentration of 30 μM resulted in a zeta potential change of only Δ13.2 mV (Table 4). An explanation for this decrease might be an inhibitory effect of CTAB in higher concentrations on IAP activity [22]. Nevertheless, usage of different CTAB concentrations facilitated a zeta potential adjustment and provides therefore flexibility for further developments with LPNEPNPP.

Selecting a CTAB concentration for following studies with LPNEPNPP was based on three aspects. First, the initial zeta potential should be negative to enable sufficient mucus permeation [5,23]. Second, the surface charge of droplets upon contact with IAP should shift to positive to support cellular uptake [9–11]. Third, the concentration of CTAB should be kept at a minimum, as cationic surfactants are well-known for their toxicity [24,25]. Consequently, LPNEPNPP containing 5 μM of CTAB (LPNEPNPP + CTAB) was chosen for further experiments, as this formulation displayed highly negatively charged droplets (−33.7 mV) and a positive zeta potential (+8.2 mV) upon incubation with IAP.

3.2. Zeta potential change over time

In the following zeta potential change of LPNEPNPP + CTAB over time was evaluated during incubation with IAP. As illustrated in Fig. 2 (left axis, ♦), a fast increase in zeta potential for LPNEPNPP + CTAB was observed within the first hour. The process then slowed down most likely caused by a reduced amount of substrate available for enzymatic cleavage. After 4 h, an overall shift in zeta potential of Δ41.9 mV was determined for LPNEPNPP + CTAB. In comparison to already established zeta potential changing NE based on different phosphorylated surfactants as listed in Table 5, the accomplished shift in zeta potential was nearly twice as high as the highest so far achieved zeta potential shift for NE highlighting the great potential of PNPP.

3.3. Phosphate release studies

3.3.1. Phosphate release studies catalyzed by isolated enzyme

In order to confirm that the zeta potential change of NEPNPP + CTAB is caused by and correlates with the cleavage of phosphate groups from PNPP, phosphate release of NEPNPP + CTAB was investigated over 4 h. Within the first hour, as shown in Fig. 2 (right axis ○), 18.7 μM of

![Fig. 2. Zeta potential as well as amount of released phosphate of NEPNPP + CTAB incubated with intestinal alkaline phosphatase over 4 h. Emulsions were prepared by diluting lipophilic preconcentrates 1:1000 (v/v) with demineralized water and adding 0.5 U·ml⁻¹ of intestinal alkaline phosphate. A zeta potential of 0 mV is illustrated by the dotted line. Indicated values are means (n = 3) ± SD.](image-url)
phosphate was released, corresponding to 64% of the overall phosphate release of 29.3 μM after 4 h. As these outcomes were in good accordance with the zeta potential change of NEPNPP+CTAB, it can be concluded that the shift in zeta potential is caused by cleavage of the phosphate group on PNPP by IAP. Overall, 92% of phosphate having been added to the formulation was cleaved off explaining why a higher zeta potential change could be achieved for NE with PNPP in comparison to POAP, as for this surfactant only 50% of phosphate moieties were cleaved off within the same period [16].

The higher phosphate release and corresponding zeta potential change determined for NEPNPP+CTAB might be explained by the PEG spacer of PNPP. It seemed due this hydrophilic linker the incorporated phosphate groups were located outside the PEG corona resulting in an improved accessibility of IAP in comparison to substance like phosphorylated tyrosine-octadecylamide synthesized by Salimi et al. [13]. Comparing PNPP to POAP, the double-tail structure of POAP could cause a steric hindrance of IAP and therefore a more pronounced phosphate release and zeta potential change was determined for NE containing the single-chain PNPP [16].

### 3.3.2. Caco-2 cell induced phosphate release

Results of phosphate release studies conducted with NEPNPP+CTAB on Caco-2 cells are illustrated in Fig. 3. With a concentration of 48.7 μM after 4 h, a pronounced phosphate release from NEPNPP+CTAB was observed. This value was significantly higher (p ≤ 0.001) compared to 24.9 μM released from the NEPNPP+CTAB in presence of PIC. However, the released amount of phosphate determined for HBS (12.5 μM) and HBS with PIC (14.3 μM) was lower than the phosphate concentration analyzed for NEPNPP+CTAB in presence of PIC. Nevertheless, NEPNPP+CTAB without PIC released 23.8 μM more phosphate compared to the reference NEPNPP+CTAB with PIC. Accordingly, upon oral administration a sufficient phosphate release at the intestinal epithelium can be anticipated providing the intended change in zeta potential.

#### Table 5

Overview of zeta potential changing nanoemulsion systems based on phosphate bearing compounds. Intestinal alkaline phosphatase (IAP).

| Phosphate bearing compound | Chemical structure | Zeta potential values before and after incubation with IAP (mV) | Maximum achieved change in zeta potential (ΔmV) | Ref. |
|---------------------------|--------------------|---------------------------------------------------------------|-----------------------------------------------|-----|
| 1,2-Dipalmitoyl-sn-glycero-3-phosphatic acid sodium | ![Chemical Structure](image) | −1.2 to 0.8 | 2.0 | [12] |
| Phosphorylated hydroxypropyl starch | ![Chemical Structure](image) | −6.5 to 1.0 | 7.5 | [15] |
| 1,2-Dipalmitoyl-sn-glycero-3-phosphatic acid sodium | ![Chemical Structure](image) | −11.2 to −3.2 | 8.0 | [26] |
| Phosphorylated serine-oleylamine | ![Chemical Structure](image) | −11.5 to −2.0 | 9.5 | [27] |
| Phosphorylated 12-amino-dodecanol | ![Chemical Structure](image) | −9.4 to 0.5 | 9.9 | [14] |
| Phosphorylated tyrosine-octadecylamide | ![Chemical Structure](image) | −12.0 to 5.3 | 17.3 | [13] |
| N,N'-bis(polyoxyethylene)oleylamine bisphosphate (POAP) | ![Chemical Structure](image) | −15.1 to 6.5 | 21.6 | [16] |
| Polyoxylethylene (9) nonylphenol monophosphate ester (PNPP) | ![Chemical Structure](image) | −33.7 to 8.2 | 41.9 |
3.4. Cell viability studies on Caco-2 cells

A resazurin assay was utilized to evaluate the impact of NE with and without PNPP+CTAB as well as unmodified PNPP on Caco-2 cell viability. As illustrated in Fig. 4, NE-PNPP+CTAB was significantly (p ≤ 0.05) more toxic in comparison to NEBlank, likely caused by the incorporated cationic surfactant CTAB that is known for its cytotoxic potential [29]. Nevertheless, with a cell viability of 70.0%, NE-PNPP+CTAB still demonstrated a low cytotoxic potential [30]. However, in comparison to other zeta potential changing systems [13, 14, 16, 27], the cationic charge is not located on the same surfactant from that the phosphate is cleaved off. Thus, cationic surfactants displaying a lower cytotoxicity compared to CTAB, such as in particular biodegradable surfactants based on arginine [31] or lysolecithin [32] could be utilized as alternatives for zeta potential changing systems based on PNPP.

Moreover, unformulated PNPP displayed no cytotoxicity up to concentrations of 0.01% (v/v) and at 0.02% (v/v) cell viability still remained at 54.3% allowing the application of higher amounts of PNPP in formulations than having been used within this study [30]. Furthermore, PNPP possessed enhanced cell compatibility in comparison to POAP, which exhibited a pronounced cytotoxic effect already at a concentration of 0.01% (v/v) and provoked almost complete die-off at 0.02% (v/v) likely caused by its cationic substructure [16].

3.5. Cellular uptake studies on Caco-2 cells

Confocal imaging was applied to evaluate the cellular uptake of NE-PNPP+CTAB including a fluorescent dye on Caco-2 cells. Fig. 5 presents a top view (A), an enlarged top view (B) and a side view (C) of the cells after treatment with NE-PNPP+CTAB. According to these images, a considerable higher cellular uptake for NE-PNPP+CTAB was found when applied without PIC. Additionally, C proves that the dye was taken up by cells and not attached on their surface. After cleavage of phosphate groups of PNPP by IAP expressed on Caco-2 cells, NE droplets should demonstrate a positive zeta potential as shown in Section 3.2 [19, 20]. As the cell membrane exhibits a dense negative charge resulting from components such as amino acids with negatively charged side chains, anionic phospholipids and heparan sulfates, positively charged carriers are able to interact more tightly with the cell surface. Thus, cell membranes are depolarized leading to an enhanced cellular uptake of these carriers. Therefore, the obtained results are in good accordance with outcomes of previous studies [6, 9–11, 33–36]. For example, Nazir et al. recently described a 2-fold enhanced uptake for zeta potential changing NE on Caco-2 cells in the absence of IAP inhibitors [27].

4. Conclusion

Zeta potential changing NE based on phosphate moiety cleavage seem to be a promising approach to improve the oral bioavailability of active pharmaceutical ingredients. In order to further improve their potency, the single-chain phosphorylated surfactant PNPP with a hydrophilic PEG linker was evaluated to address the issue of steric enzyme hindrance caused by a PEG corona around the oily droplet core. By incorporating PNPP and the cationic surfactant CTAB, a NE with a distinctly improved zeta potential change from negative to positive in comparison to previous systems was generated. Furthermore, a more efficient cleavage of phosphate moieties was achieved and an enhanced...
cellular uptake was observed underlining the potential of this new concept.

Based on these results, PNPP is the so far most promising tool for developing zeta potential changing NE containing phosphorylated substances. Therefore, follow-up studies investigating the properties of such a system upon encapsulation of suitable drugs would be of great interest in the future.

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CRediT authorship contribution statement

Markus Kurpiers: Methodology, Writing - original draft, Visualization, Formal analysis. Julian Dominik Wolf: Conceptualization, Methodology, Validation, Writing - review & editing. Christian Steinbring: Investigation. Sergey Zaichik: Investigation. Andreas Bernkop-Schnürch: Supervision, Project administration, Funding acquisition, Writing - review & editing.

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.

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