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The Role of Emulsification and Composition in Modulating Cell Type-Dependent Cytotoxicity in o/w nanoemulsion

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Research Article

Keywords: cytotoxicity, nanoemulsion, MTT assay, L929, Caco-2

Posted Date: March 10th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8833895/v1

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Additional Declarations: No competing interests reported.

Abstract

Background

Nanoemulsions (NEs) are emerging as effective drug delivery systems for poorly water-soluble drugs, enhancing solubility and bioavailability. However, concerns regarding their cytotoxicity persist, and systematic evaluations across different cell types remain limited.

Methods

NE–Et and NE–PEG were prepared with identical compositions except for the cosurfactant. Cytotoxicity was evaluated using standard viability assays at full-strength (100%) and at actual NE concentrations (NE%). Individual components, binary and ternary mixtures, and complete NEs were tested. The effect of dilution medium (deionized water vs. DMEM) on NE cytotoxicity was examined.

Results

Both NEs displayed concentration-dependent cytotoxicity. In Caco-2 cells, individual surfactants and almond oil exhibited high cytotoxicity, whereas complete NEs demonstrated higher cell viability (~ 47–64% at NE%), indicating the protective effects of emulsification. Ethanol- and PEG400-based mixtures showed composition-dependent responses. In L929 cells, surfactant-containing mixtures were most toxic, while oil–PEG and oil–water systems maintained moderate to high viability (~ 60–70% at NE%). No significant differences in cytotoxicity were observed between deionized water (DI) and DMEM-diluted NEs.

Conclusions

Emulsification mitigates the cytotoxicity of individual NE components. Both the type of cosurfactant and formulation composition critically affect cell-specific responses. These results highlight the importance of rational cosurfactant selection and component organization in designing safe and biocompatible NE-based drug delivery systems.

Background

Nanoemulsions (NEs) are increasingly recognized as versatile systems for drug delivery. They are biphasic dispersions of two immiscible liquids, typically oil and water, in which nanosized droplets of one phase are dispersed within the other. NEs improve the solubility and bioavailability of poorly water-soluble drugs, making them suitable for oral, topical, and intravenous administration. Oil-in-water (o/w) NEs, for instance, contain oil droplets dispersed in a continuous aqueous phase and often include triglycerides, vegetable oils, or other lipophilic excipients (1, 2).

Evaluating cytotoxicity is crucial to ensure the biocompatibility of nano-formulations. The MTT assay is a widely used method for this purpose (3–5). It measures mitochondrial metabolic activity rather than direct cell viability, as mitochondrial enzymes convert the yellow MTT dye into purple formazan crystals, which are quantifiable spectrophotometrically (6). Figure 1 shows a schematic representation of the MTT assay process.

Despite their advantages, NEs can be cytotoxic due to their surfactant and cosurfactant content. Conventional surfactants, such as polysorbate 80 (Tween80), may trigger anaphylactoid reactions and tissue damage (7). The toxicity of NEs is not solely determined by surfactant type but also by droplet organization, interfacial structure, and molecular interactions (8). High surfactant concentrations can disrupt cell membranes, alter lipid organization, and interfere with mitochondrial function. Surfactant monomers can impair membrane barrier properties even below the critical micellar concentration (9), disrupt mitochondrial membranes, and inhibit essential cellular processes such as steroidogenesis (10–12). Organic solvents, such as ethanol, and specific oils can also contribute to cytotoxicity in a dose- and time-dependent manner (13, 14).

However, when researchers prepare nanoemulsions and evaluate their cytotoxicity, they often encounter inconsistent and sometimes contradictory results. Different cytotoxicity assays can yield divergent outcomes even for seemingly identical formulations (15). The observed inconsistencies stem from multiple interrelated factors, including surfactant chemical structure, concentration, interactions with cellular membranes, and potential disruption of mitochondrial activity (15). Such variability complicates the interpretation of safety data and underscores the urgent need for systematic, rigorous screening of nanoemulsion components to mitigate unpredictable toxic effects (16).

Given these challenges, a more mechanistic and comparative approach is required to understand the origins of NE-related cytotoxicity. In this study, two NE formulations with identical component ratios were prepared, differing only in their cosurfactant type ethanol or polyethylene glycol 400 (PEG400). This controlled variation allows direct assessment of how subtle compositional differences influence toxicity and nanoscale organization. Cytotoxicity was systematically analyzed not only for the complete NEs but also for their individual components and for binary and ternary mixtures, enabling deconvolution of the physicochemical factors contributing to overall toxicity.

To provide a broader biological perspective, both human intestinal epithelial (Caco-2) and murine fibroblast (L929) cells were used as representative models of epithelial and connective tissues, respectively. This comparative evaluation clarifies why similar nanoemulsion formulations can yield divergent cytotoxicity outcomes and identifies key parameters governing safe and predictable NE design. Ultimately, this work aims to elucidate the factors underlying contradictory cytotoxicity trends reported for NEs, propose formulation-based strategies to minimize toxicity, and highlight approaches for rational design of biocompatible nanoemulsion systems.

Figure 1. **Schematic representation of the MTT assay process.**

Methods

This study aimed to investigate the cytotoxicity of two NE formulations—ethanol-containing (NE–Et) and PEG400-containing (NE–PEG)—and their individual components, mixtures, and complete emulsions in human intestinal epithelial (Caco-2) and murine fibroblast (L929) cells. The influence of dilution medium on cytotoxicity was also assessed.

Materials

Caco-2 and L929 cell lines (Pasteur Institute of Iran) were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and 0.05% trypsin-EDTA (Bioidea, Iran). Almond oil (Sigma-Aldrich), Tween80, Span80 (CDH, India), PEG400, and ethanol (Neutron, Iran) were used for NE preparation.

NE Preparation

The nanoemulsion composition and excipient concentrations used in this study were adopted from our previously published work, in which the formulation was systematically optimized for physical stability and biocompatibility. In the present study, this validated formulation was employed to specifically investigate the impact of co-solvent type (ethanol versus PEG400) on physicochemical properties and cytotoxicity, rather than to re-optimize formulation parameters (17).

Two NEs were prepared using ethanol (NE–Et) or PEG400 (NE–PEG) as cosurfactants. Almond Oil, Tween80, and Span80 (6:1 ratio) were mixed, followed by the addition of the cosurfactant and deionized water. Formulations contained 3% oil, 35% surfactant mix, 12% ethanol, and 50% water (NE–Et), or 2% oil, 33% surfactant mix, 15% PEG400, and 50% water (NE–PEG). The formulations were assessed for transparency, droplet size, and stability.

Stability and Size Analysis

Stability was evaluated through centrifugation (10,000 rpm, 30 min), three freeze–thaw cycles (–20 to 25°C), and 30-day storage at 25°C (18). Droplet size and polydispersity index (PDI) were measured by dynamic light scattering (DLS), Scatteroscope1, K-One, Korea. Morphology was observed using transmission electron microscopy (TEM), ZEISS LEO 906 E, Germany, after phosphotungstic acid staining.

MTT Assay

Caco-2 and L929 cells were seeded (1×10^3 cells/well, 96-well plates) and treated with NEs or their components. After 6 h (Caco-2) or 48 h (L929), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/mL) was added and incubated for 3 h. The resulting formazan was dissolved in

DMSO, and absorbance was measured at 570 nm (19–23). Cell viability was normalized to untreated controls.

±, whereas L929 fibroblasts were incubated for 48 h in accordance with standard cytotoxicity screening protocols for normal cells.

Statistical Analysis

Results are expressed as mean ± standard deviation (SD) of at least three experiments. Statistical significance was determined using one-way ANOVA (analysis of variance) with Tukey's post-hoc test or Student's t-test ($p < 0.05$, GraphPad Prism 10.4.2).

Results

Visual inspection showed that both formulations were optically transparent immediately after preparation. After one month of storage, NE–PEG exhibited a slight reduction in transparency, whereas NE–Et maintained its transparent appearance, and no visible phase separation was observed in either formulation (Figure S1).

The median droplet diameter (D50) of the nanoemulsions was first evaluated using DLS, as summarized in Table 1. No statistically significant difference was observed between the initial droplet sizes of NE–Et and NE–PEG ($p > 0.05$).

DLS analysis indicated particle sizes within the acceptable nanoscale range, supporting the short-term physical stability of both formulations (18, 19). Transmission electron microscopy (TEM, Fig. 2) confirmed spherical droplet morphology, in agreement with the DLS measurements. Both formulations demonstrated short-term stability suitable for in vitro assays.

Then, the MTT assays demonstrated concentration-dependent cytotoxicity for both ethanol-based (NE–Et) and PEG400-based (NE–PEG) NEs and their excipients in Caco-2 and L929 cells.

Table 1
Zeta potential (mV), polydispersity index (PDI), and median droplet diameter (D50, nm) of the nanoemulsions before and after stability tests, obtained from DLS

NEs	Zeta potential (mV)	PDI	D50 (fresh, nm)	D50 (after centrifugation, nm)	D50 (after freeze–thaw cycles, nm)	D50 (after long-term storage, nm)
NE-Et	-13.87 ± 0.6	0.10 ± 0.1	11.2 ± 1.0	8.22 ± 1.0	13.8 ± 1.3	12.7 ± 2.0
NE-PEG	-5.2 ± 0.81	0.08 ± 0.1	14.7 ± 1.5	13.3 ± 2.5	15.1 ± 1.6	296 ± 23

Cytotoxicity of NEs

As illustrated in Fig. 3a–b, both Caco-2 and L929 cells exhibited concentration-dependent viability responses to the nanoemulsions. In Caco-2 cells (Fig. 3a), at 100% concentration, both formulations markedly reduced cell viability, with NE–Et showing approximately 15% and NE–PEG approximately 4.5% survival. Upon dilution to 50% with DI water, viability increased to around 34% for NE–Et and 35% for NE–PEG, both dilutions with no significant difference between formulations ($p > 0.05$). The highest viability was observed at 20% dilution, reaching about 64% for NE–Et and 47% for NE–PEG ($p > 0.05$). At lower dilutions (10% and 5%), both formulations again exhibited moderate toxicity, with viabilities ranging from 35% to 47%, and again, no statistically significant differences between the two groups.

In L929 fibroblasts (Fig. 3b), both NE–Et and NE–PEG exhibited strong, concentration-independent cytotoxicity at higher concentrations. At 100%, 50%, and 20% dilutions, viability remained below 2% for both formulations. The highest viability appeared at 10% dilution (~ 70% for NE–Et and ~ 60% for NE–PEG), while moderate toxicity persisted at 5% (~ 36% and ~ 31%, respectively).

Cytotoxicity of NE individual components

To evaluate the contribution of individual excipients (almond oil, Tween80, Span80, ethanol, PEG400, and DI water), cytotoxicity was examined in both cell lines (Fig. 4a–b).

In Caco-2 cells (Fig. 4a), almond oil showed high cytotoxicity, reducing cell viability to ~ 6% at 100% and ~ 31% at NE%. Tween80 and Span80 were strongly cytotoxic at 100% (~ 18% and 27% viability, respectively) and slightly toxic at NE% (~ 67% and 70%). Ethanol and PEG400 were minimally cytotoxic, maintaining viabilities of ~ 68% and 93% at 100% and ~ 99% at NE%, respectively. DI water also caused moderate reductions in viability (~ 67% at 100% and ~ 75% at NE%).

In L929 cells (Fig. 4b), the same pattern was largely preserved, though the relative sensitivities differed. Almond oil and PEG400 were minimally toxic (~ 77% and 87% viability at 100%, increasing to ~ 94% and 99% at NE%, respectively). Tween80 was the most cytotoxic (< 2% at both concentrations). Span80 and ethanol showed moderate, concentration-dependent effects (~ 46% and 52% at 100%, increasing to ~ 84% and 80% at NE%), and DI water caused a moderate and concentration-independent reduction (~ 44% at 100% and ~ 60% at NE%). Collectively, almond oil emerged as the most cytotoxic excipient in Caco-2 cells, whereas Tween80 showed the highest cytotoxicity in L929 fibroblasts, while PEG400 consistently showed excellent biocompatibility.

Binary mixtures of NE components

Binary mixtures exhibited distinct cytotoxicity profiles across both cell types. In Caco-2 cells (Fig. 5), surfactant-based combinations (surf–PEG, surf–Et, and surf–DI) were the most cytotoxic, whereas a mixture of surfactants and oil (surf–oil) markedly reduced toxicity, increasing viability to ~ 83% at 100% and ~ 85% at NE%. Other oil-containing mixtures, including oil–DI, oil–PEG, and oil–Et, were highly cytotoxic at 100% (2–37%) but less toxic at NE%, particularly oil–PEG, which maintained ~ 86% viability.

A similar trend was seen in L929 fibroblasts (Fig. 6). Surfactant-containing binary mixtures (surf, surf–Et, surf–PEG, surf–DI, and surf–oil) showed strong cytotoxicity (1–2% viability at both concentrations), while other combinations demonstrated moderate to high survival (> 48%). Thus, in both cell lines, the presence of surfactant dominated cytotoxic effects, though the degree of recovery at NE% was somewhat greater in Caco-2 than in L929.

Ternary mixtures of NE components

Ternary mixtures also exhibited characteristic cytotoxicity patterns in both cell types (Fig. 7). Surfactant–oil–cosurfactant systems (surf–oil–Et and surf–oil–PEG) were highly cytotoxic (< 2% viability at both concentrations), whereas surf–oil–DI was equally toxic at 100% (< 2%) but showed significant recovery at NE% (~ 69%, $p < 0.0001$). Systems containing oil–DI–Et and oil–DI–PEG showed strong, concentration-dependent recovery, increasing from ~ 1.8% and ~ 2.9% at 100% to ~ 68% and ~ 97% at NE%, respectively ($p < 0.0001$).

The pattern was generally comparable in Caco-2 cells, where surf–oil–DI, surf–oil–Et, and surf–oil–PEG were among the most cytotoxic, while oil–DI–Et and oil–DI–PEG showed greater viability, particularly at NE%. Both NE–Et and NE–PEG maintained moderate viability in the NE% range (~ 70% and ~ 60%), significantly higher than several non-emulsified ternary mixtures ($p < 0.0001$).

Effect of dilution medium

To assess the influence of the dilution medium, NEs were diluted with either DI water or culture medium (DMEM). As shown in Supplementary Figures S1 and S2, no significant difference in cytotoxicity was observed between dilution media in either cell line or at any tested concentration. This finding suggest that the observed cytotoxicity originates primarily from the intrinsic formulation properties, rather than from interactions with external medium components.

Discussion

The cytotoxicity assessment of ethanol-based (NE–Et) and PEG400-based (NE–PEG) nanoemulsions (NEs) and their respective components revealed that both formulations exhibited concentration-dependent cytotoxicity, but the magnitude and pattern of response varied between the two cell lines. When evaluated comparatively, the emulsified forms (NE–Et and NE–PEG) consistently showed lower toxicity than their corresponding binary and ternary precursor mixtures in both Caco-2 and L929 cells, suggesting that nano-encapsulation effectively mitigates the intrinsic cytotoxicity of individual components through reduced membrane contact, encapsulation within nanoscale droplets, and improved interfacial stability (20, 21). These results align with previous studies on nanoemulsion-based systems, where nanoscale encapsulation enhanced bioavailability and reduced uncontrolled cytotoxicity through more regulated cell–component interactions (8, 22, 23) .

In parallel with cytotoxicity evaluation, physicochemical characterization of the nanoemulsions provided important insights into their stability and biological performance. Both formulations exhibited low polydispersity indices (PDI < 0.1), indicating narrow droplet size distributions and homogeneous systems (24). The zeta potential values were relatively low in magnitude (− 13.87 mV for NE–Et and − 5.2 mV for NE–PEG), which is characteristic of non-ionic nanoemulsions stabilized predominantly by steric rather than electrostatic mechanisms (25, 26). In particular, the lower absolute zeta potential of NE–PEG can be attributed to PEG-induced charge shielding at the droplet interface, which reduces measurable surface charge without necessarily compromising colloidal stability (27, 28).

Despite the weak electrostatic repulsion, both nanoemulsions maintained nanoscale droplet sizes under centrifugation and freeze–thaw stress, supporting the dominant role of steric stabilization. However, NE–PEG showed a pronounced increase in median droplet diameter after long-term storage, indicating a gradual increase in droplet aggregation or partial coalescence over time. This behavior may be associated with PEG rearrangement at the oil–water interface and reduced interfacial rigidity during prolonged storage (26, 29). Importantly, this size increase did not translate into proportionally increased cytotoxicity, indicating that droplet size alone was not the primary determinant of cellular response in the tested concentration range.

Comparative cellular responses

Although both cell types displayed concentration-dependent viability changes, their sensitivity patterns reflected their biological origin. Caco-2, an intestinal epithelial-like and cancer-derived line, exhibited higher tolerance to surfactants but greater sensitivity to oil-rich compositions, whereas L929 fibroblasts, a normal dermal cell model, were more susceptible to surfactant-induced membrane perturbation but less sensitive to lipid-based toxicity. These differences likely stem from distinct membrane lipid compositions, metabolic activities, and oxidative stress levels between cancerous epithelial and normal fibroblast cells (30, 31). In essence, while emulsification mitigated cytotoxicity across both models, the dominant toxic driver differed — oil and osmotic imbalance in Caco-2 versus surfactant content in L929.

The relative resistance of Caco-2 cells to surfactants can be attributed to the structural features of their epithelial membrane. Tight junction proteins, including claudins-1, -3, -4, and − 7, occludin, and junctional adhesion molecule-A (JAM-A), are associated with cholesterol-rich membrane domains that contribute to barrier stability (32). Cholesterol is critical for maintaining tight junction integrity, and its depletion has been shown to markedly reduce transepithelial electrical resistance (33). Although surfactants such as sodium dodecyl sulfate can transiently disrupt epithelial membranes, the cholesterol-enriched architecture of Caco-2 cells provides partial protection against irreversible membrane damage (34), consistent with the relatively preserved viability observed in this study.

In contrast, the enhanced sensitivity of Caco-2 cells to oil-rich formulations likely arises from their altered lipid metabolism and redox balance. Caco-2 cells exhibit modulation of fatty acid receptor and transporter expression, reflecting increased lipid uptake (35). Proliferative and immature Caco-2 cells

have been shown to be particularly vulnerable to lipid peroxide-induced injury due to limited glutathione-dependent detoxification capacity (36), and cancer cells' reliance on lipid uptake and β-oxidation further increases their susceptibility to oxidative stress and lipotoxicity (37, 38). These mechanisms provide a plausible explanation for the oil-driven cytotoxicity observed in Caco-2 cells.

L929 fibroblasts are widely recognized as a sensitive model for evaluating membrane damage and surfactant-mediated cytotoxicity. Their susceptibility to surfactant-induced membrane disintegration has been consistently demonstrated across fibroblast-based toxicity assays and wound dressing evaluations (39–41), which aligns with the pronounced reduction in L929 viability observed at increasing surfactant concentrations in the present formulations.

Finally, the overall reduction in cytotoxicity upon emulsification can be explained by physicochemical sequestration mechanisms. Emulsification limits direct oil–cell contact and reduces the availability of free surfactant monomers. Lytic agents can be incorporated into lipid emulsion cores, thereby preventing direct membrane interaction (42), and high-molecular-volume oils further reduce membrane solubilization within aggregated systems (43). Consistent with these findings, microemulsions have been shown to exhibit substantially lower cytotoxicity than neat surfactants or lipids (44), supporting the protective effect of emulsification observed in this study.

Effect of individual components

Among the individual ingredients, Tween80 exhibited the highest cytotoxicity in L929 fibroblasts, consistent with its well-documented surfactant-induced membrane-disruptive effects at elevated concentrations (45). In contrast, almond oil induced the most pronounced cytotoxicity in Caco-2 cells, highlighting the greater sensitivity of this cancer-derived epithelial model to lipid-rich components.

Span80 also demonstrated notable toxicity, albeit slightly lower than Tween80. In contrast, PEG400 maintained high cell viability in both lines, consistent with its established biocompatibility and steric stabilization role (46, 47). Almond oil produced divergent effects — highly cytotoxic in Caco-2 but minimally so in L929 — implying cell-specific lipid metabolism or uptake pathways (48). DI water unexpectedly caused moderate toxicity in both models, which can be attributed to osmotic stress and hypo-osmotic swelling (49, 50). Ethanol was only mildly cytotoxic, and its inclusion in NE–Et did not significantly exacerbate toxicity, suggesting that ethanol's contribution to overall cytotoxicity was secondary compared to surfactant effects.

Binary and ternary mixtures: comparative insights

Binary and ternary component mixtures provided additional insight into how formulation composition influences cytotoxicity across cell types. Binary systems containing surfactants, particularly the surfactant–PEG, surfactant–Et, and surfactant–DI combinations, exhibited the strongest cytotoxicity in both models. However, the surfactant–oil combination markedly reduced toxicity, with viabilities exceeding 80% at NE%, indicating that the coexistence of oil and surfactant favors the formation of partial micellar or emulsion-like structures that limit direct membrane interaction. This trend was

consistent across both Caco-2 and L929, though fibroblasts appeared more sensitive to residual free surfactant molecules, possibly due to weaker adaptive mechanisms to membrane fluidization (51).

PEG-containing mixtures, such as oil–PEG, consistently maintained higher viabilities (> 85%) in both cell lines, supporting the hypothesis that PEG provides a steric shield and reduces surface charge-driven membrane disruption (46, 47). Conversely, DI-containing mixtures caused pronounced viability loss, particularly in Caco-2, likely from osmotic stress rather than chemical toxicity. Interestingly, DI–Et mixtures in both lines exhibited improved viability, suggesting ethanol's partial mitigation of osmotic imbalance (50).

Ternary mixtures generally showed similar patterns: systems containing surfactant-oil-cosurfactant (e.g., surf–oil–PEG or surf–oil–Et) were highly cytotoxic in both cell types, while the inclusion of DI water (e.g., surf–oil–DI or oil–DI–PEG) reduced toxicity at NE%. The relatively lower toxicity of these DI-containing systems at NE% supports the idea that increased dilution and partial micelle formation can decrease the effective concentration of free surfactant molecules. Moreover, the complete NEs demonstrated significantly higher viabilities than corresponding ternary systems, indicating that the optimized emulsified state — rather than merely the presence of the same components — governs biocompatibility.

Formulation and concentration effects

Cytotoxicity in both cell lines followed a non-linear, concentration-dependent trend. At intermediate dilutions (e.g., 20%), viability was highest, whereas very concentrated or highly diluted systems both exhibited greater toxicity. This phenomenon likely arises from structural changes in droplet aggregation, altered component distribution, or changes in osmotic pressure at extreme dilutions. Previous studies have reported similar non-linear viability curves in nanoemulsions, influenced by droplet size, triglyceride content, and interfacial film stability (8, 52). Collectively, the results indicate that both too-high and too-low NE concentrations disturb the equilibrium between emulsion integrity and cell compatibility.

Medium and methodological considerations

No significant difference in cytotoxicity was observed when NEs were diluted with DI water versus culture medium, in either cell line. This suggests that the intrinsic physicochemical features of the formulations, rather than interactions with serum proteins or medium components, predominantly determined cellular responses (44). The MTT assay, while widely used, carries inherent limitations in the context of nanoscale systems, as dilution can alter colloidal organization. Consequently, the apparent cytotoxicity may not fully represent the behavior of intact nanoemulsions. Complementary methods, such as impedance-based real-time cell analysis or live/dead fluorescence imaging, would provide a more accurate picture of true cytocompatibility (5, 44).

Overall interpretation

Integrating data across both cell models highlights several consistent principles. Surfactants remain the primary determinants of cytotoxicity, with their concentration and availability in free form directly correlating with reduced cell viability. Oil–PEG and oil–DI systems exhibit higher compatibility due to

reduced surfactant content and better interfacial balance. The emulsified NEs (NE–Et and NE–PEG) showed substantially lower cytotoxicity than their precursor mixtures, confirming that nanoscale organization decreases the bioavailability of toxic molecular species and limits direct membrane perturbation.

Importantly, the comparative behavior of Caco-2 and L929 cells underscores the necessity of optimizing NE formulations for diverse biological barriers: epithelial cells are more sensitive to lipid and osmotic stress, while fibroblasts are more vulnerable to surfactant-mediated disruption. Therefore, achieving biocompatibility across tissue types requires fine-tuning of surfactant ratios, co-solvent type, and droplet stability. Altogether, these findings affirm that controlling surfactant concentration, promoting molecular compatibility, and ensuring stable nanoscale assembly are key strategies for designing safe, cyto-compatible NE-based delivery systems.

Conclusion

The cytotoxicity of ethanol- and PEG400-based nanoemulsions (NEs) was primarily governed by surfactant concentration and formulation architecture. Fully emulsified systems exhibited substantially lower cytotoxicity than their binary and ternary precursors, confirming the protective effect of nanoscale encapsulation. Formulations containing PEG400 and oil demonstrated superior biocompatibility, whereas surfactant-rich mixtures remained the most cytotoxic. Moderate dilution enhanced cell viability, and distinct responses of Caco-2 and L929 cells reflected their inherent physiological differences. Overall, precise control of surfactant levels and stabilization of nanoscale organization are critical for the rational design of safe and biocompatible NE-based drug delivery systems.

List of abbreviations

NEs; Nanoemulsions

NE%; Nanoemulsion concentrations

Tween 80; Polysorbate 80

PEG 400; Polyethylene glycol 400

NE–Et; Nanoemulsion formulations containing ethanol

NE–PEG; Nanoemulsion formulations containing polyethylene glycol 400

DMEM; Dulbecco's Modified Eagle Medium

PDI; Polydispersity index

DLS; Dynamic light scattering

TEM; Transmission electron microscopy

MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

SD; Standard deviation

D50; Median droplet diameter

Declarations

Ethics approval and consent to participate;

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests

Funding

This study was funded by Tehran University of Medical Sciences, School of Advanced Technologies in Medicine. The funder had no role in the design of the study, data collection, analysis, interpretation of data, or in writing the manuscript.

Authors' contributions

A. A. conceived and designed the study concept. N. K. acquired, analyzed, and interpreted the data, performed the statistical analysis, and drafted the manuscript. A. A. critically revised the manuscript for important intellectual content. M. P. L. and N. K. provided administrative, technical, and material support. N. K. and A. A. supervised the overall study. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to express their sincere gratitude to Tehran University of Medical Sciences for providing the research facilities, scientific support, and administrative assistance that made this study possible.

References

1. Bai L, Huan S, Gu J, McClements DJ. Fabrication of oil-in-water nanoemulsions by dual-channel microfluidization using natural emulsifiers: Saponins, phospholipids, proteins, and polysaccharides. Food Hydrocolloids. 2016;61:703–11.
2. Wilson RJ, Li Y, Yang G, Zhao C-X. Nanoemulsions for drug delivery. Particuology. 2022;64:85–97.
3. Seyedhassantehrani N, Karimi R, Tavoosidana G, Amani A. Concurrent study of stability and cytotoxicity of a novel nanoemulsion system–an artificial neural networks approach. Pharm Dev Technol. 2017;22(3):383–9.
4. Hashemi-Hafshejani S, Amani A, Azami SJ, Valian HK, Mohebali M, Salimi M, et al. Nanoemulsion of spiramycin against tachyzoites of Toxoplasma gondii, RH strain: preparation, toxicology, and efficacy studies. Iran J Public Health. 2023;52(7):1495.
5. Oroojalian F, Mohammadzadeh T, Ebrahimzadeh A, Alesheikh P, Shafiei R, Amani A. Black seed oil nanoemulsion containing albendazole against protoscoleces of Echinococcus granulosus: An in vivo study on C57BL/6 mice. Iran J Basic Med Sci. 2025;28(6):755.
6. Ghasemi M, Turnbull T, Sebastian S, Kempson I. The MTT assay: utility, limitations, pitfalls, and interpretation in bulk and single-cell analysis. Int J Mol Sci. 2021;22(23):12827.
7. Yoosuf S, Nambiar P. Microbial biosurfactant based nanoemulsions and their applications: A review. J Surfactants Deterg. 2024;27(2):163–81.
8. Wooster TJ, Moore SC, Chen W, Andrews H, Addepalli R, Seymour RB, et al. Biological fate of food nanoemulsions and the nutrients they carry–internalisation, transport and cytotoxicity of edible nanoemulsions in Caco-2 intestinal cells. RSC Adv. 2017;7(64):40053–66.
9. Partearroyo MA, Ostolaza H, Goñi FM, Barberá-Guillem E. Surfactant-induced cell toxicity and cell lysis: A study using B16 melanoma cells. Biochem Pharmacol. 1990;40(6):1323–8.
10. Levine S, Han Z, Liu J, Farmer D, Papadopoulos V. Disrupting mitochondrial function with surfactants inhibits MA-10 Leydig cell steroidogenesis. Cell Biol Toxicol. 2007;23(6):385–400.
11. Kononenko V, Erman A, Petan T, Križaj I, Kralj S, Makovec D, et al. Harmful at non-cytotoxic concentrations: SiO2-SPIONs affect surfactant metabolism and lamellar body biogenesis in A549 human alveolar epithelial cells. Nanotoxicology. 2017;11(3):419–29.
12. Findlay RD, Taeusch HW, David-Cu R, Walther FJ. Lysis of red blood cells and alveolar epithelial toxicity by therapeutic pulmonary surfactants. Pediatr Res. 1995;37(1):26–30.
13. Neuman MG, Haber JA, Malkiewicz IM, Cameron RG, Katz GG, Shear NH. Ethanol signals for apoptosis in cultured skin cells. Alcohol. 2002;26(3):179–90.
14. Jamalzadeh L, GHAFOORI H, Sariri R, Rabuti H, Nasirzade J, Hasani H et al. Cytotoxic effects of some common organic solvents on MCF-7, RAW-264.7 and human umbilical vein endothelial cells. 2016.
15. Nikolić I, Đoković J, Mehn D, Guerrini G, Savić S, Jordan O et al. When conventional approach in toxicity assays falls short for nanomedicines: a case study with nanoemulsions. Drug Delivery

Translational Res. 2025:1–19.

16. Azeem A, Rizwan M, Ahmad FJ, Iqbal Z, Khar RK, Aqil M, et al. Nanoemulsion components screening and selection: a technical note. AAPS PharmSciTech. 2009;10(1):69–76.
17. Ketabchi N, Esmaeili F, Amani A. Pharmacokinetic Characteristics and Therapeutic Efficacy of Frankincense Extract Nanoemulsion on Multiple Sclerosis: An Animal Study. BioNanoScience. 2025;15(3):1–15.
18. Devalapally H, Zhou F, McDade J, Goloverda G, Owen A, Hidalgo IJ, et al. Optimization of PEGylated nanoemulsions for improved pharmacokinetics of BCS class II compounds. Drug Delivery. 2015;22(4):467–74.
19. Caputo F, Clogston J, Calzolai L, Rösslein M, Prina-Mello A. Measuring particle size distribution of nanoparticle enabled medicinal products, the joint view of EUNCL and NCI-NCL. A step by step approach combining orthogonal measurements with increasing complexity. J Controlled Release. 2019;299:31–43.
20. Fraga M, Laux M, Rejane dos Santos G, Zandona B, Dos Santos Giuberti C, De Oliveira M, et al. Evaluation of the toxicity of oligonucleotide/cationic nanoemulsion complexes on Hep G2 cells through MTT assay. Die Pharmazie-An Int J Pharm Sci. 2008;63(9):667–70.
21. Liu D-Z, Lecluyse EL, Thakker DR. Dodecylphosphocholine-mediated enhancement of paracellular permeability and cytotoxicity in Caco-2 cell monolayers. J Pharm Sci. 1999;88(11):1161–8.
22. Demisli S, Mitsou E, Pletsa V, Xenakis A, Papadimitriou V. Development and study of nanoemulsions and nanoemulsion-based hydrogels for the encapsulation of lipophilic compounds. Nanomaterials. 2020;10(12):2464.
23. Gonçalves RF, Martins JT, Abrunhosa L, Vicente AA, Pinheiro AC. Nanoemulsions for enhancement of curcumin bioavailability and their safety evaluation: Effect of emulsifier type. Nanomaterials. 2021;11(3):815.
24. Ahmad Farooqui DN, Baghel MK, Salman MM, Kumar MP. Formulation and evaluation of nanoemulsion for topical drug delivery system. Int J Pharm Res Appl. 2025.
25. Kulkarni NS, Ranpise NS, Rathore DS, Dhole S, editors. Characterization of Self-Microemulsifying Dosage Form: Special Emphasis on Zeta Potential Measurement2019.
26. Tran E, Richmond GL. Interfacial Steric and Molecular Bonding Effects Contributing to the Stability of Neutrally Charged Nanoemulsions. Langmuir. 2021.
27. Devalapally H, Zhou F, McDade JE, Goloverda GZ, Owen AJ, Hidalgo IJ, et al. Optimization of PEGylated nanoemulsions for improved pharmacokinetics of BCS class II compounds. Drug Delivery. 2015;22:467–74.
28. Tayeb HH, Piantavigna S, Howard CB, Nouwens AS, Mahler SM, Middelberg APJ, et al. Insights into the interfacial structure-function of poly(ethylene glycol)-decorated peptide-stabilised nanoscale emulsions. Soft Matter. 2017;13 43:7953–61.
29. Alayoubi A, Alqahtani S, Kaddoumi A, Nazzal S. Effect of PEG surface conformation on anticancer activity and blood circulation of nanoemulsions loaded with tocotrienol-rich fraction of palm oil.

AAPS J. 2013;15(4):1168–79.

30. Abdulateef SA, Al-Saffar AZ, Sulaiman GM, Mohammed HA, Al Ali A, Al Shmrany H. Enhanced therapeutic efficacy of Tamoxifen against breast cancer using extra virgin olive oil-based nanoemulsion delivery system. Nanatechnol Reviews. 2025;14(1):20250217.
31. McClements DJ, Rao J. Food-grade nanoemulsions: formulation, fabrication, properties, performance, biological fate, and potential toxicity. Crit Rev Food Sci Nutr. 2011;51(4):285–330.
32. Lambert D, O'Neill C, Padfield P. Methyl-β-cyclodextrin increases permeability of Caco-2 cell monolayers by displacing specific claudins from cholesterol rich domains associated with tight junctions. Cell Physiol Biochem. 2007;20(5):495–506.
33. Lambert D, O'NEILL CA, Padfield PJ. Depletion of Caco-2 cell cholesterol disrupts barrier function by altering the detergent solubility and distribution of specific tight-junction proteins. Biochem J. 2005;387(2):553–60.
34. Anderberg EK, Artursson P. Epithelial transport of drugs in cell culture. VIII: Effects of sodium dodecyl sulfate on cell membrane and tight junction permeability in human intestinal epithelial (Caco-2) cells. J Pharm Sci. 1993;82(4):392–8.
35. Berger E, Nassra M, Atgié C, Plaisancié P, Géloën A. Oleic acid uptake reveals the rescued enterocyte phenotype of colon cancer Caco-2 by HT29-MTX cells in co-culture mode. Int J Mol Sci. 2017;18(7):1573.
36. Cepinskas G, Kvietys PR, Aw TY. ω3-Lipid peroxides injure CaCo-2 cells: Relationship to the development of reduced glutathione antioxidant systems. Gastroenterology. 1994;107(1):80–6.
37. Cuvelier G, Vermonden P, Rousseau J, Feron O, Rezsohazy R, Larondelle Y. Resistance to CLnA-induced ferroptosis is acquired in Caco-2 cells upon differentiation. Front Cell Death. 2023;2:1219672.
38. Rolver MG, Severin M, Pedersen SF. Regulation of cancer cell lipid metabolism and oxidative phosphorylation by microenvironmental acidosis. Am J Physiology-Cell Physiol. 2024;327(4):C869–83.
39. Chen R, Salisbury AM, Percival SL. In vitro cellular viability studies on a concentrated surfactant-based wound dressing. Int Wound J. 2019;16(3):703–12.
40. Moreno JJ. Arachidonic acid release and prostaglandin E2 synthesis as irritant index of surfactants in 3T6 fibroblast cultures. Toxicology. 2000;143(3):275–82.
41. Shen C, Jiang L, Long X, Dahl KN, Meng Q. Cells with higher cortical membrane tension are more sensitive to lysis by biosurfactant di-rhamnolipids. ACS Biomaterials Sci Eng. 2019;6(1):352–7.
42. Jumaa M, Müller BW. Lipid emulsions as a novel system to reduce the hemolytic activity of lytic agents: mechanism of the protective effect. Eur J Pharm Sci. 2000;9(3):285–90.
43. Warisnoicharoen W, Lansley AB, Lawrence MJ. Toxicological evaluation of mixtures of nonionic surfactants, alone and in combination with oil. J Pharm Sci. 2003;92(4):859–68.

44. Bu P, Narayanan S, Dalrymple D, Cheng X, Serajuddin AT. Cytotoxicity assessment of lipid-based self-emulsifying drug delivery system with Caco-2 cell model: Cremophor EL as the surfactant. Eur J Pharm Sci. 2016;91:162–71.
45. Munir R, Lisec J, Swinnen JV, Zaidi N. Lipid metabolism in cancer cells under metabolic stress. Br J Cancer. 2019;120(12):1090–8.
46. Sabuncu AC, Kalluri BS, Qian S, Stacey MW, Beskok A. Dispersion state and toxicity of mwCNTs in cell culture medium with different T80 concentrations. Colloids Surf B. 2010;78(1):36–43.
47. Strachan JB, Dyett BP, Nasa Z, Valery C, Conn CE. Toxicity and cellular uptake of lipid nanoparticles of different structure and composition. J Colloid Interface Sci. 2020;576:241–51.
48. Hu X, Liao M, Shen K, Ding K, Campana M, van der Kamp S, et al. Unraveling How Membrane Nanostructure Changes Impact the Eye Irritation of Nonionic Alkyl Ethoxylate Surfactants. ACS Appl Mater Interfaces. 2023;15(50):59087–98.
49. HAMZELOO MM, Taiebi N, Mosaddegh M, Eslami TB, Esmaeili S. The effect of some cosolvents and surfactants on viability of cancerous cell lines. 2014.
50. Rachmawati H, Novel MA, Ayu S, Berlian G, Tandrasasmita OM, Tjandrawinata RR, et al. The in vitro–in vivo safety confirmation of peg-40 hydrogenated castor oil as a surfactant for oral nanoemulsion formulation. Sci Pharm. 2017;85(2):18.
51. Crowston JG, Healey PR, Hopley C, Neilson G, Milverton EJ, Maloof A. Water-mediated lysis of lens epithelial cells attached to lens capsule. J Cataract Refractive Surg. 2004;30(5):1102–6.
52. Czajkowska-Kośnik A, Wolska E, Chorążewicz J, Sznitowska M. Comparison of cytotoxicity in vitro and irritation in vivo for aqueous and oily solutions of surfactants. Drug Dev Ind Pharm. 2015;41(8):1232–6.

Figures



Figure 1

Schematic representation of the MTT assay process.

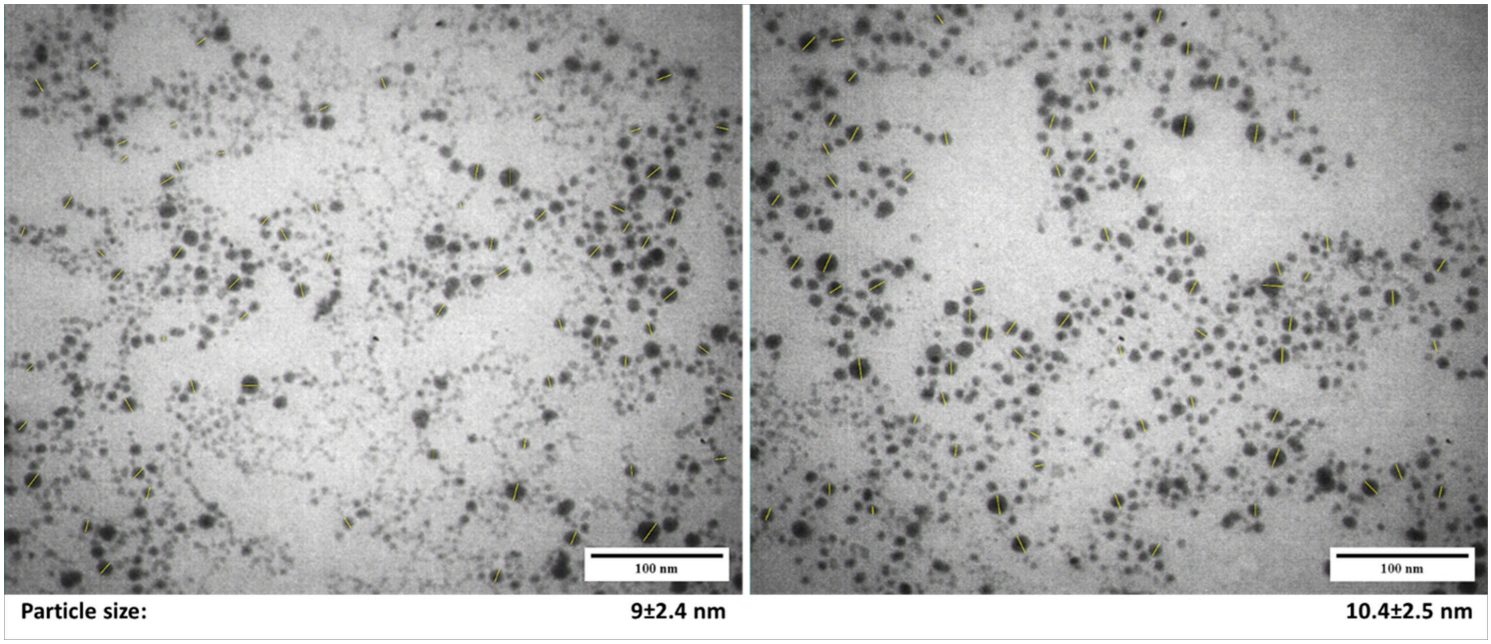


Figure 2

TEM images. The left picture is captured from NE-Et, and the right from NE-PEG. The mean particle size measured using ImageJ software is indicated below each image.

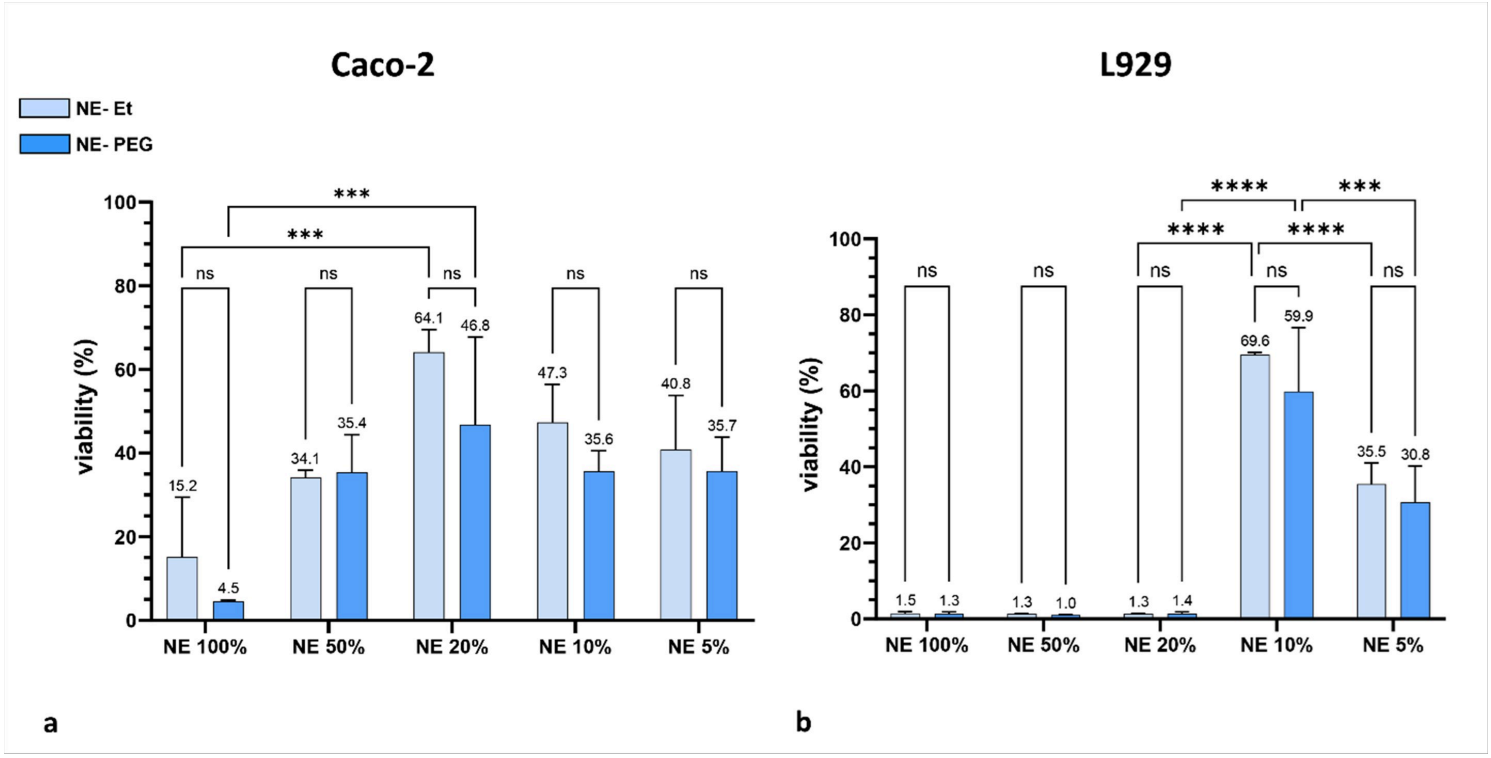


Figure 3

Viability (%) of (a) Caco-2 and (b) L929 cells assessed by MTT assay at different dilutions of the nanoemulsions NE–Et and NE–PEG (containing 12% ethanol and 15% PEG400, respectively). Data were analyzed by ordinary one-way ANOVA with Tukey's test (*p < 0.05, **p < 0.01, ***p < 0.001).

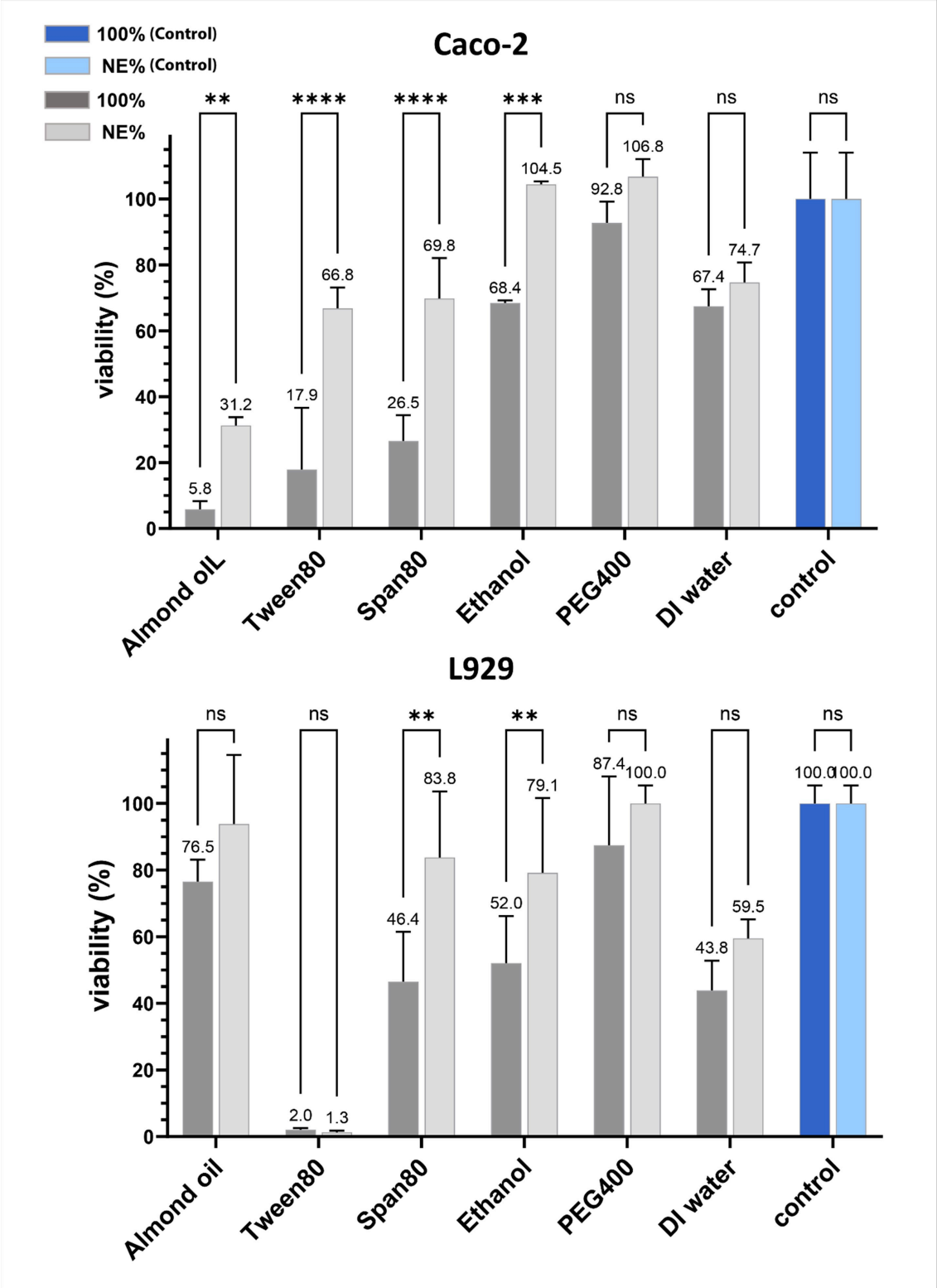


Figure 4

Viability assessment (MTT assay) of individual NE components in (a) Caco-2 and (b) L929 cells. Data were analyzed by one-way ANOVA with Tukey's post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). the components are assigned in gray and the control group in blue.

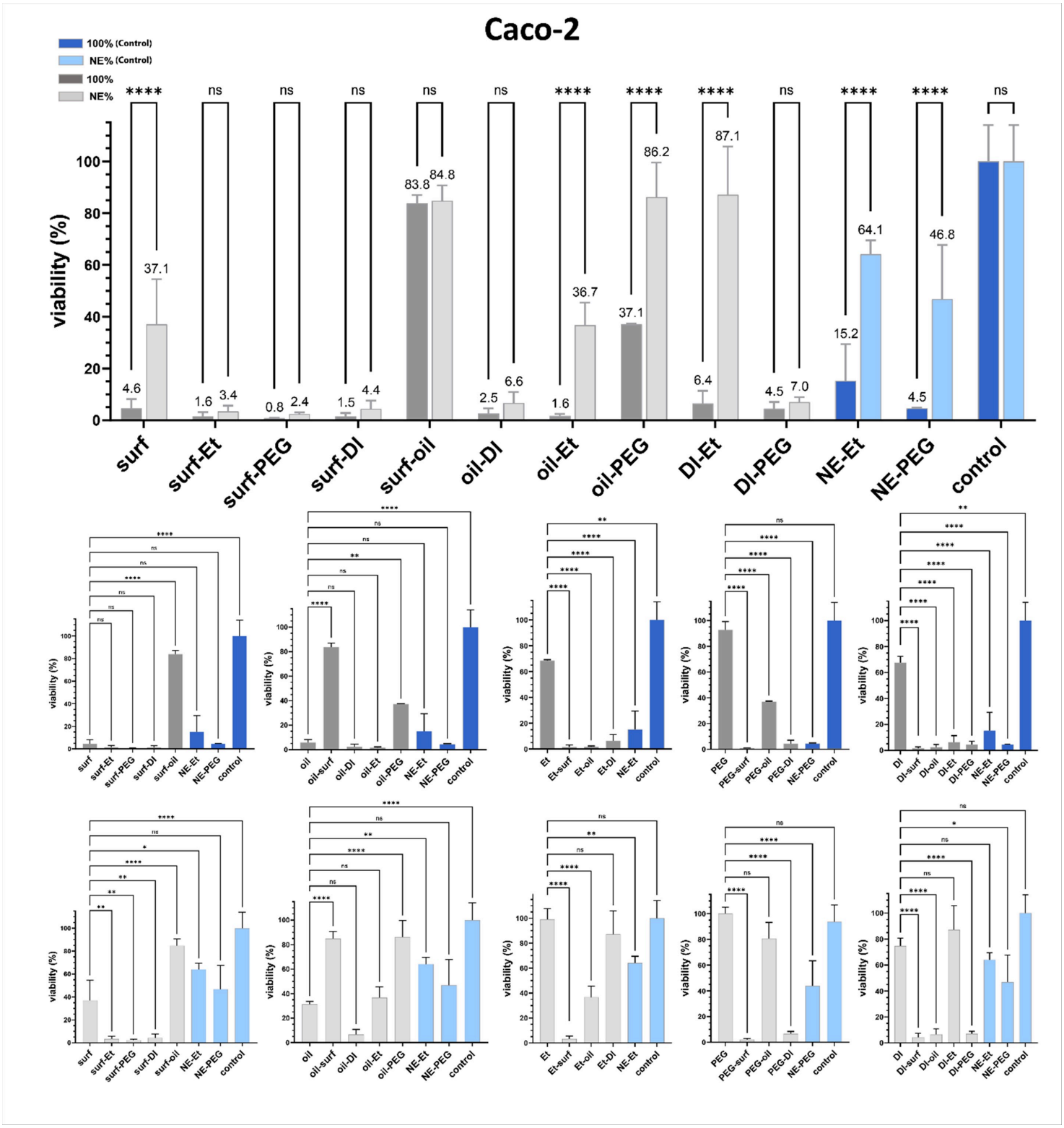


Figure 5

Caco-2 cells viability assessment (MTT assay) of the NEs components in pairs. Data was analyzed by one-way ANOVA with Tukey's post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Top row: All components are shown as binary at 100% values compared to the actual NE concentration (NE%), other binary compositions, and both complete NEs. Middle row: from left to right, see Surfactants (surf), almond oil (oil), Ethanol (Et), PEG400 (PEG), and deionized water (DI) effect on Caco-2 cells viability in

100% values in comparison with other relevant binary groups, both NEs and control. Bottom row: from left to right, see surf, oil, Et, PEG, and DI effect on Caco-2 cells viability in NE% values in comparison with other relevant binary groups, both NEs and control. NEs and control are assigned by blue and the others by gray.

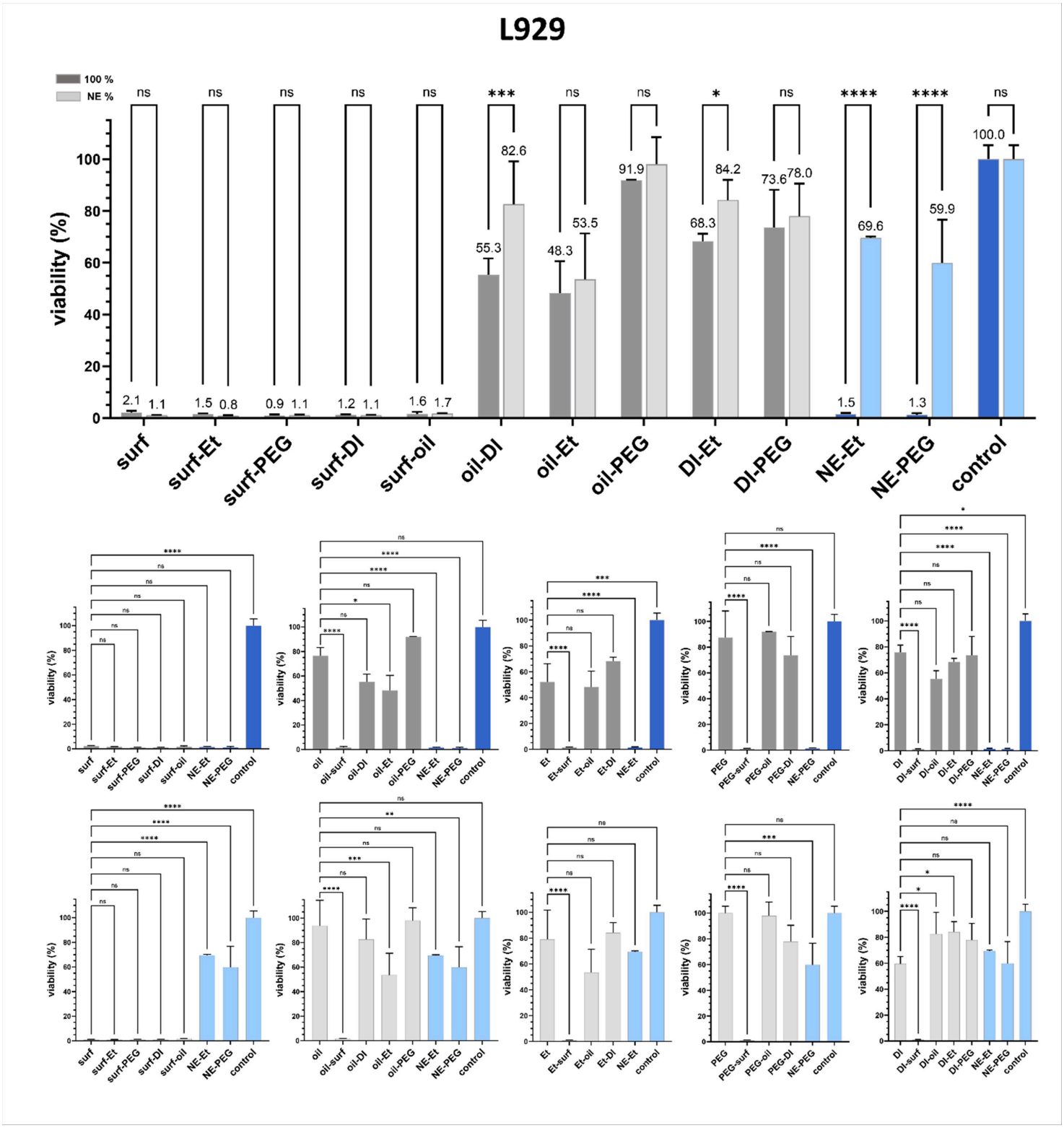


Figure 6

L929 cells viability assessment (MTT assay) of the NEs components in pairs. Data was analyzed by one-way ANOVA with Tukey's post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Top row: All components are shown as binary at 100% values compared to the actual NE concentration (NE%), other binary compositions, and both complete NEs. Middle row: from left to right, see Surfactants (surf), almond oil (oil), Ethanol (Et), PEG400 (PEG), and deionized NE (DI) effect on Caco-2 cells viability in 100% values in comparison with other relevant binary groups, both NEs and control. Bottom row: from left to right, see surf, oil, Et, PEG, and DI effect on Caco-2 cells viability in NE% values in comparison with other relevant binary groups, both NEs and control. NEs and control are assigned by blue and the others by gray.

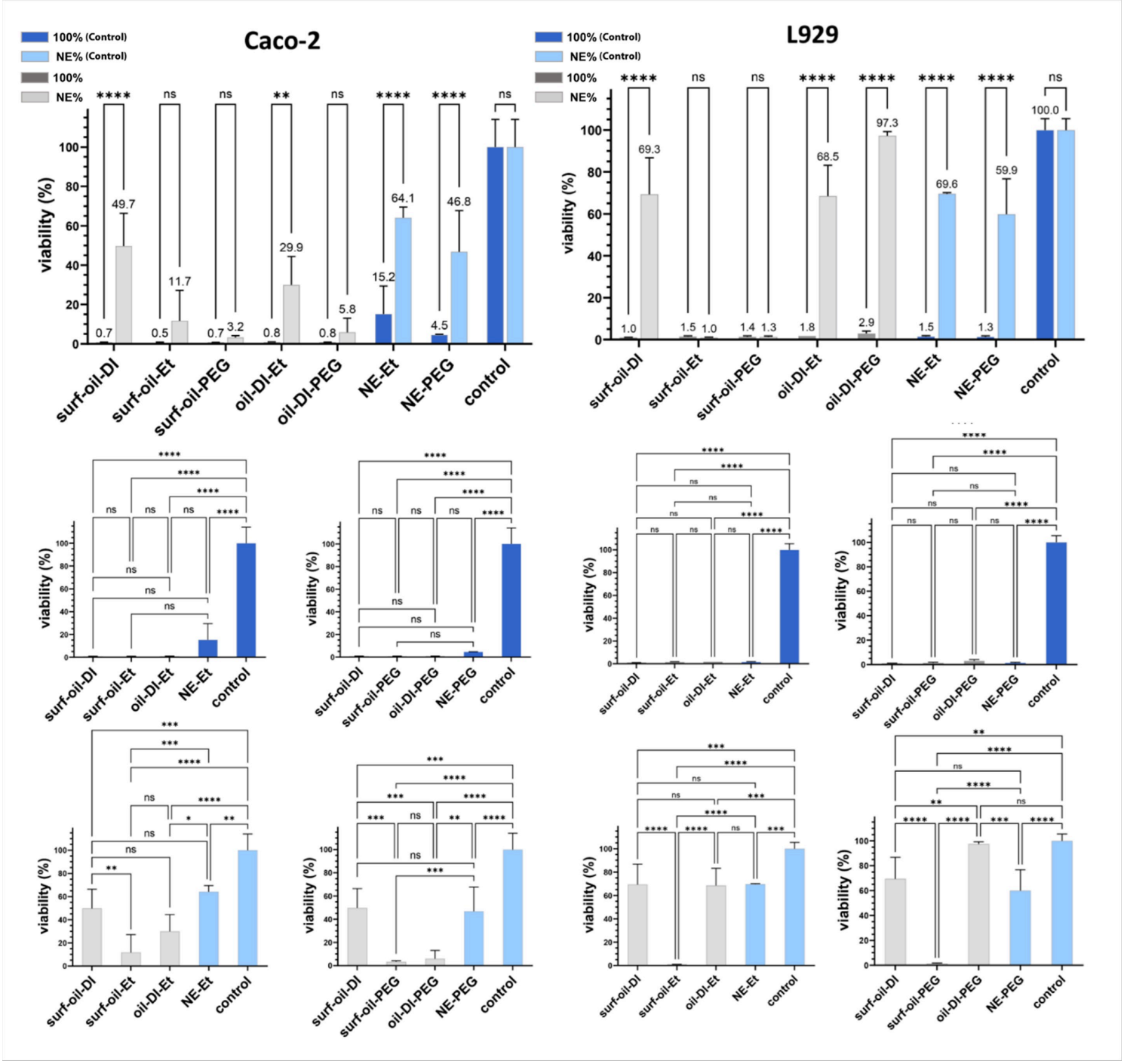


Figure 7

Caco-2 cells (the left side) and L929 cells (the right side) viability assessment (MTT assay) of the NEs components in triplets. Data was analyzed by one-way ANOVA with Tukey's post hoc test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001). Top row: All components are shown as binary at 100% values compared to the actual NE percentage (NE%), other binary compositions, and both complete nanoemulsions. Middle row: from left to right, see Surfactants (surf), almond oil (oil), Ethanol (Et), PEG400 (PEG), and deionized NE (DI) effect on Caco-2 cells viability in 100% values in comparison with other relevant binary groups, both NEs and control. Bottom row: from left to right, see surf, oil, Et, PEG, and DI effect on Caco-2 cells viability in NE% values in comparison with other relevant binary groups, both NEs and control. NEs and control are assigned by blue and the others by gray.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- FigureS1.png
- FigureS1.pdf.png
- FigureS3.png