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Surfactant dependent toxicity of lipid nanocapsules in HaCaT cells

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ABSTRACT

Lipid nanocapsules (LNC) have been suggested for a variety of pharmaceutical applications. Among them approaches for drug delivery to the skin appear particularly interesting. The current standard composition has been modified to better understand their properties by selecting a variety of different surfactants. LNC have been prepared using different non-ionic surfactants (Solutol® HS15: Polyoxyl 15 Hydroxystearate; Cremophor® EL: Polyoxyl 35 Castor Oil; Simulsol® 4000: Polyoxyl 40 Hydrogenated Castor Oil; Vitamin E TPGS®: alpha-tocopheryl poly(ethylene glycol) succinate; Polysorbate 20 and 80) and analysed for their size, stability, drug release and toxicity on keratinocytes in cell culture. The feasibility of LNC using different surfactant was surprisingly easy and led to a variety of stable formulations that were selected for further investigations. Surfactants led to a variability of the release kinetics (t50% release varied from Polysorbate 20: 2.5 h to Simulsol® 4000: 5.0 h), however different formulations from the same surfactant did not differ significantly. In vitro toxicity of LNC was surfactant type dependent and a correlation between LNC and the pure respective surfactant was found. This toxicity was found to be mainly independent from the surface active properties. The surfactant type in LNC is easily interchangeable from formulation point of view. LNC appear to be appropriate as carrier for cutaneous delivery however toxicity can vary distinctly depending on the surfactant used for the preparation.

1. Introduction

Nanoparticles and nanoemulsions have been investigated for diverse applications, among these mainly the improvement of the bioavailability of drugs (Zara et al., 1999; Chen et al., 2001; Peltier et al., 2006) and drug targeting (Lamprecht et al., 2002; Lu et al., 2008). They are attracting attention as they can provide improved therapeutic regimes for existing drug molecules on the skin. Especially, lipid nanocarriers have been used as drug carrier systems for cutanous applications.

Different drug classes were proposed and investigated such as anti-inflammatory drugs (Miyazaki et al., 2003), vitamins (Duclairoir et al., 2003), or bactericides (Dillen et al., 2004). It has been described that drug loaded nanoparticles could achieve sustained release and consequently improve the therapeutic effect of skin preparations (Luengo et al., 2006; Lboutounne et al., 2002). Another advantage is the possibility to protect sensitive drugs through their encapsulation into these nanoparticles (Wu et al., 2009). Some studies showed that nanoparticles can improve the generally low skin penetration and produce targeting to some skin layers such as the epidermis or skin appendages (Sivaramakrishnan et al., 2004; Otberg et al., 2004; Abdel-Mottaleb and Lamprecht, 2011).

For lipophilic drugs, different lipid nanocarriers are known, which entrap the drug molecules in their lipid cores. Examples for such lipid nanocarriers are solid lipid nanoparticles (SLN), nanostructured lipid carriers (NLC), and lipid nanocapsules (LNC). SLN consist of a solid lipid core which is often stabilized by an external monolayer of steric or charged surfactant (Husseini and Pitt, 2008). SLN, however, suffer from several drawbacks such as limited drug loading, risk of gelation and leakage of the drug during storage caused by lipid polymorphism (Joshi and Patravale, 2008). NLC are composed of a mixture of solid lipids with liquid lipids (oils) leading to an overall melting point depression but with keeping the solid state at body temperature (Müller et al., 2002). LNC are drug carriers in the nanometer range and are prepared by phase inversion of an emulsion (Heurtault et al., 2002). These nanocapsules are composed of a medium chain triglyceride core surrounded by a capsule shell made from hydrophilic and lipophilic surfactants. Due to their very small size in the range of 25–100 nm and their capacity to encapsulate lipophilic and hydrophilic drugs, LNC can be an excellent alternative to liposomes, emulsions, or microemulsions for pharmaceutical applications (Lamprecht et al., 2004). As an alternative to the firstly introduced surfactant Solutol® HS15 a

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Table 1
Chemical composition of the surfactants.

Surfactant	Main hydrophilic Part	Main hydrophobic part
Cremophor® EL	PEG_{35}	Triricinoleat
Solutol® HS15	PEG_{15}	Hydroxystearate
Vitamin E TPGS®	PEG_{23}	Alpha-tocopheryl succinate
Simulsol® 4000	PEG_{40}	Hydrogenated castor oil
Polysorbate 20	PEG_{20}	Monolaurate
Polysorbate 80	PEG_{20}	Monooleate

variety of other non-ionic surfactants are of interest for feasibility purposes and other properties such as stability and drug release. LNC have been evaluated for transdermal drug delivery and have led to promising data (Abdel-Mottaleb and Lamprecht, 2011).

Due to the postulated capsule structure with an inner oil core, the general stability in terms of integrity of the structure is potentially lower than with other lipid carrier types. This can lead to a higher importance of single components sine they may act separately once the capsule disintegrates.

Since LNC are composed from a relative high amount of surfactant and subsequently its toxicity may play a major role for its therapeutic potential. In consequence, the choice of type and quantity of surfactant may influence this aspect essentially having a significant influence on the applicability.

The aim of this work was to elucidate the influence of using different surfactants in the preparation of LNC for cutaneous formulations, to obtain a first set of data on the toxicity and find potential explanations.

2. Materials and methods

2.1. Materials

Ibuprofen, Cremophor® EL (Polyoxyl 35 Castor Oil) and Solutol® HS15 (Polyoxyl 15 Hydroxystearate) were kind samples from BASF (Ludwigshafen, Germany), Vitamin E TPGS® (d-alpha-tocopheryl poly(ethylene glycol) Succinate 1000) was a kind gift from Kodak-Eastman, and Simulsol® 4000 (Polyoxyl 40 Hydrogenated Castor Oil) was a kind gift from Seppic (France). Polysorbate 20 and 80 were purchased from Prolabo (France). Surfactant structures are described in Table 1. Miglyol® 812 (medium chain triglyceride; MCT) was from Fagron GmbH (Barsbüttel, Germany). Lipoid® S75-3 (soybean lecithin) was a kind gift from Lipoid GmbH (Ludwigshafen, Germany). Spectra/Por® dialysis membrane, molecular weight cut off 12,000–14,000 Da was purchased from Spectrum Laboratories Inc., Rancho Dominguez, Canada. All other chemicals were of analytical grade or equivalent purity.

2.2. Preparation of lipid nanocapsules

The preparation of lipid nanocapsules was based on solvent free phase inversion method that allows the preparation of very small nanocapsules by thermal manipulation of oil/water system (Lamprecht et al., 2004). Briefly, an ibuprofen amount equivalent to 4% (w/w) was dissolved in the internal oily triglyceride phase MCT prior to all preparation steps by magnetic stirring for 5 min. The oil phase (low: 26%, high: 21%) was then mixed with the respective surfactant (low: 30%, high: 45%), distilled water (low: 40%, high: 30%), sodium chloride (90 mg), and soybean lecithin (45 mg) were also added to give a total weight of 5 g. The mixture was heated under magnetic stirring up to 85 °C (until a distinct drop of conductivity occurs) to ensure that the phase inversion temperature was passed and a w/o emulsion was formed. Then the emulsion was cooled to 55 °C. During the cooling, another complete phase inversion to an o/w emulsion occurs. This cycle was repeated twice before adding 5 ml of distilled water at 4 °C. The LNC suspension was then stirred for 10 min before further analysis.

2.3. Measurement of particle size of lipid nanocapsules

The LNC were analysed for their particle size and size distribution in terms of the average volume diameters and polydispersity index by photon correlation spectroscopy using a particle size analyser (Brookhoven Instruments Corporation, New York, USA) at a fixed angle of 90° at 25 °C. The nanocapsules suspension was diluted with distilled water before analysis and samples were analysed in triplicate.

2.4. Determination of in vitro drug release kinetics

The study was carried out using a modified USP dissolution apparatus II as described elsewhere (Abdel-Mottaleb and Lamprecht, 2011). After fixation to the apparatus, the tubes were immersed in the dissolution vessel which contained 100 ml of the release medium (Sorenson phosphate buffer pH 7.4). The glass baskets were rotated at 25 rpm and aliquots each of 3 ml were withdrawn from the release medium at predetermined time points. The samples were assayed spectrophotometrically for ibuprofen content at λ_{max} = 264 nm using online UV spectrophotometer and the concentration of the drug was determined from a previously constructed calibration curve.

2.5. LNC toxicity test on HaCat cells

HaCaT cells were seeded in 96-well plates (approximately 10,000 cells per well) and grown in Dulbecco's Modified Eagle Medium (DMEM). Thereafter, cells were incubated with the various preparations at different dilutions for 24 h. After carefully removing the supernatant, the cell viability was determined with the MTT test. Cytotoxicity was expressed as percentage of controls (untreated cells) against concentration in mg/ml. The expression as molar concentration did seemed to be useful since several surfactants are mixtures of different components. All experiments were repeated six times.

2.6. Tensiometry study

Surface tension as a function of time was measured using a DSA30 drop shape analysis system (Kruss, Germany). Briefly, surface tension was determined by bubble shape analysis in real time. Air bubble was immerged into a optical glass bowl containing the solution of single surfactants at 25 °C. Surfactant solutions were prepared with Milli-Q water at 0.001 and 0.01 mg/ml.

3. Results

At higher concentrations hardly any difference in surface tension at the triglyceride–water interface was observed at lower concentrations the surface tension decreased in the order Cremophor < Polysorbate < Simulsol < Solutol (Fig. 1, Table 2).

The replacement of Solutol as the "standard" by different non-ionic surfactants let to slight changes in LNC diameter (data not shown) however did not influence the feasibility zone of LNC. As a consequence two comparable formulations of mass equivalency per surfactant were retained. These compositions were kept constant for all further evaluations. At the higher surfactant concentration particle size was comparable at around 50 nm for all surfactants used and lowering the surfactant concentration still allowed to keep the mean particle diameter in the range of 100–150 nm, except for the cases using Polysorbates (Table 3). All formulations were stable over 3 months at 4 °C.

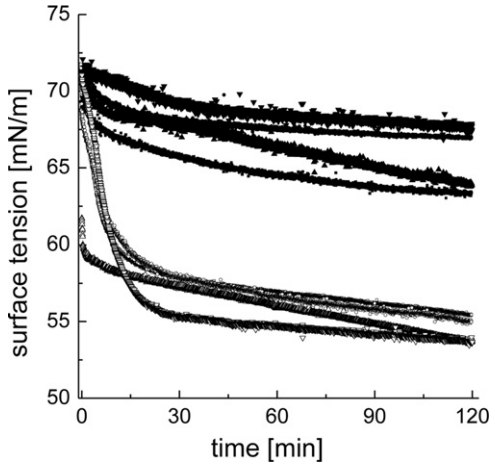

Fig. 1. Adsorption kinetic at air/water interface of Solutol® HS15 (▼, ▽) Cremophor® EL (■, □), Simulsol (●, ○) and Polysorbate 80 (▲, △) at 0.001 mg/ml (full) and 0.01 mg/ml (empty).

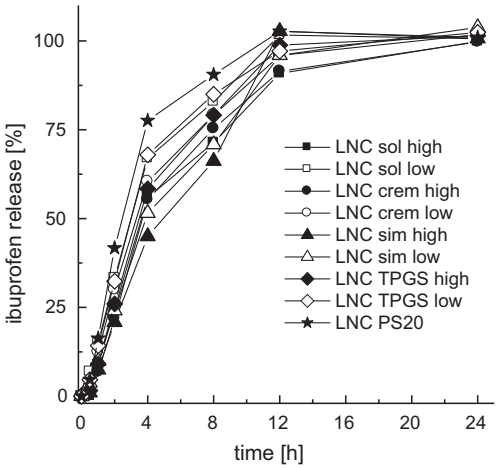

Fig. 2. Cumulated ibuprofen release versus time from LNC in phosphate buffer of pH 6.8 ($n = 3$; data are shown as mean ± SD).

Table 2
Surface active properties of the various surfactants.

Surfactant	Surface tension [mN/m]		HLB
	0.001 mg/ml	0.01 mg/ml	
Solutol® HS15	68.5	54.5	14–16
Cremophor® EL	64.4	56.7	12–14
Simulsol® 4000	67.4	56.4	14–16
Vitamin E TPGS®	–	–	~13
Polysorbate 20	–	–	16.7
Polysorbate 80	66.2	55.8	15.0

In vitro drug release of the model drug ibuprofen led to almost complete release after 12 h in phosphate buffer pH 7.4 (Fig. 2). The choice of surfactant influenced the drug release significantly and while Polysorbate resulted in the fastest release with over 75% released ibuprofen after 4 h while Simulsol was slowest with 40–50% release over the same period. However, ibuprofen release was not dependent on the surfactant concentration. A f2-test comparison between formulations of the high and low surfactant concentration, respectively, led to the result that there was no statistical difference in any of these cases (data not shown).

Tests in cultured HaCaT cells exhibited distinct differences in toxicity with pure surfactants where an order could be established TPGS1500 > Solutol® HS15 > Polysorbate 20 > Polysorbate 80 > Cremophor® EL > Simulsol® 4000 (Fig. 3).

When cellular toxicity was expressed against surfactant concentrations used in the LNC formulations, all toxicity results for one surfactant type were similar (Fig. 4). The presence of ibuprofen in the LNC formulations did not essentially alter the cell viability. Again LNC toxicity was highly surfactant dependent and data were comparable with the outcome of the tests with pure surfactant. On the other hand, particle size did not influence the toxicity in a significant way. It was remarkable that linear correlation between

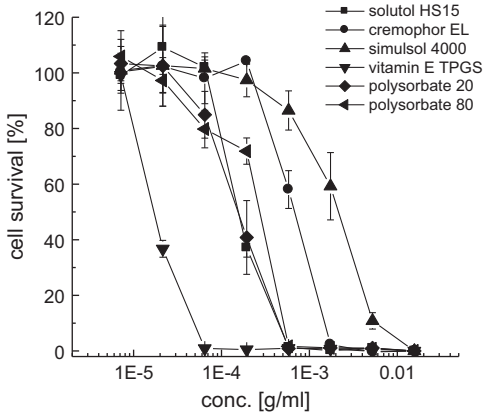

Fig. 3. Different non-ionic surfactants in solution of similar concentrations were tested for their toxicity to HaCaT cells after incubation for 8 h ($n = 6$; data are shown as mean ± SD).

both toxicities was found and that concentrations at IC50 of LNC were slightly lower than for the pure surfactants (Fig. 5, Table 4).

4. Discussion

Due to their small size LNC require a relatively high amount of surfactants in order to stabilize their very large surface (Lamprecht et al., 2002). It is known that the surfactant, in the first studies mainly Solutol® HS15 is leaking with time from the LNC surface into the surrounding aqueous media (Lamprecht and Benoit, 2006). When applying the various surfactants feasibility and stability of LNC were comparable which surely related to the similar surface tension obtained with the various surfactants. No correlation between surface active properties and the in vitro release was found

Table 3
LNC particle size with different surfactants ($n = 3$).

Surfactant	45% Particle size [nm]	30% Particle size [nm]
Solutol® HS15	48 ± 5	95 ± 9
Cremophor® EL	50 ± 5	102 ± 7
Simulsol® 4000	58 ± 1	145 ± 24
Vitamin E TPGS®	54 ± 10	102 ± 14
Polysorbate 20	–	242 ± 4
Polysorbate 80	–	233 ± 5

Table 4
IC50 of the different surfactants and the respective LNC formulations ($n = 6$).

Surfactant	IC50	
	Pure surfactant [$\times 10^{-5}$ mg/ml]	LNC
Solutol® HS15	17.0 ± 0.8	7.9 ± 0.7
Cremophor® EL	66.7 ± 4.2	84.8 ± 3.1
Simulsol® 4000	188.0 ± 31.4	131.0 ± 6.1
TPGS1500	1.87 ± 0.1	2.9 ± 0.2
Polysorbate 20	17.3 ± 2.4	13.6 ± 1.1
Polysorbate 80	23.4 ± 1.0	38.5 ± 3.1

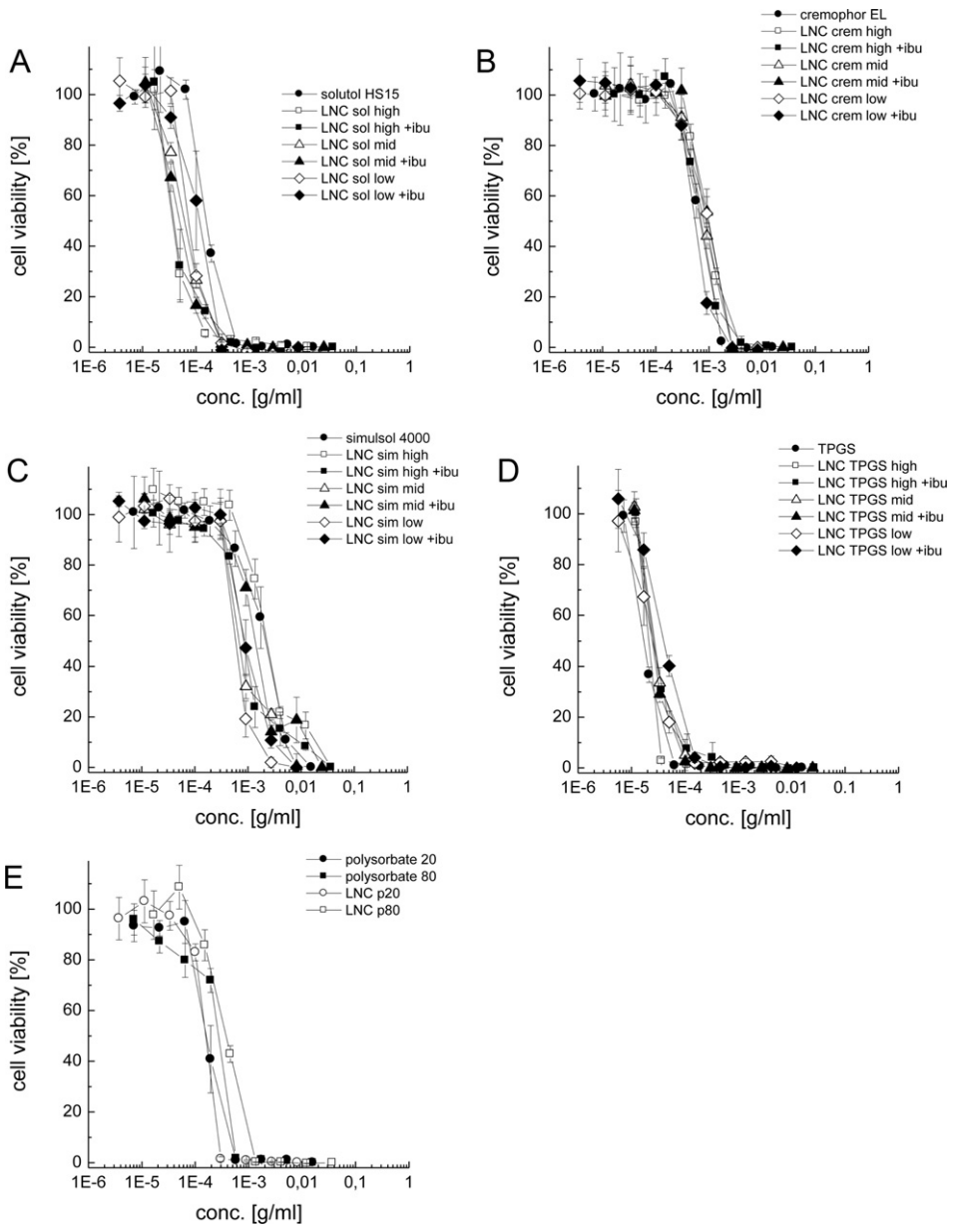

Fig. 4. Blank and drug loaded LNC prepared with different non-ionic surfactants of similar concentrations were tested for their toxicity to HaCaT cells after incubation for 8 h ($n = 6$; data are shown as mean ± SD). LNC concentrations were normalized to the actual surfactant content of each preparation in order to allow direct comparison.

however reasons for this observation are still unclear. The surfactant concentration-independent release of ibuprofen could be explained by observations from a recent study where drug release from LNC was triggered by the lipid matrix and barely by the surfactants on the corona (Abdel-Mottaleb et al., 2010). However, surfactant apparently still plays a certain role in drug release by governing surface properties of the nanocarriers and by solubilizing the released drug by the freely available surfactant in the release medium. Especially influences by the PEG chain properties could be possible. Besides, ibuprofen release was not influenced by the size of LNC (Table 3) despite a higher exchange surface with the dissolution medium for the smallest nanocapsules. Thus, release mechanism could be explained rather by a destabilisation of LNC at 37 °C. This hypothesis is in agreement with a study on LNC showing a limited stability of LNC in simulated intestinal fluid (Roger et al., 2009). Similarly, we observed a limited integrity of LNC on the skin when tested in Franz-cell experiments (Abdel-Mottaleb and Lamprecht, 2011). Therefore, in vitro toxicity testing is essential in order to identify potential differences between formulations and the surfactant alone.

The discovered correlation between surfactant and LNC toxicity seems not to be surprising since all other remaining components (e.g. triglycerides, lecithin, etc.) are considered as non-toxic. Additionally, similar IC50 values between LNC and pure surfactants are likely due to the release of surfactant from the LNC corona due the destabilisation mechanism discussed above.

Other physicochemical properties seem to have minor influence such as the particle size which had no influence on the cellular toxicity. Especially the recent conclusion that LNC lose their integrity in the skin may suggest that surfactants will be available in the intracellular compartments as a rather free molecule after the administration of surfactants or respective LNC.

Even when dealing with non-ionic surfactants, the toxicity aspect is something to address due to the high surfactant concentrations present in the formulation. Toxicity levels were significantly different between non-ionic surfactants. Viability studies

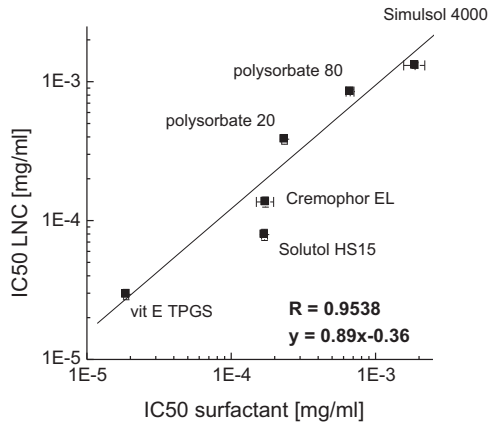

Fig. 5. Correlation between IC50 values obtained with each surfactant type for pure compound and as LNC formulation, respectively. LNC concentration was calculated on the basis of actual surfactant content.

showed IC50 of Solutol® HS15 was eleven fold lower than Simulsol® 4000. These results were in accordance with other related studies (Woodcock et al., 1992). The authors showed that integrity of cells was preserved after incubation of Cremophor® EL at 1:10 while lysis of cells was observed with Polysorbate 80 and Solutol® HS15 at 1:100. The toxic effect of surfactants is mainly explained by their amphiphilic structure. The hydrophilic and lipophilic parts interact with polar head groups and lipophilic tails of cellular lipid bilayer respectively, resulting in a disruption of the plasma membrane (Partearroyo et al., 1990). Thus, variations of toxicity between surfactants may be explained by their surface activity. However, this hypothesis is apparently too much simplified since the tensiometry study revealed similar surface properties between surfactants in the same concentration range as viability experiments. Surface tensions of Solutol® HS15, Polysorbate 80, Simulsol® 4000 and Cremophor® EL were very similar at both 0.001 and 0.01 mg/ml after 2 h-adsorption at the air/water interface. Another hypothesis linked to the chemical nature of excipients may be more advanced. Toxicity of non-ionic surfactants has been reported to decrease inversely with increasing hydrophilic chain length and proportional to the size of the lipophilic part (Ernst and Arditti, 1980). In accordance with previous discussion, long hydrophilic chains could prevent the destabilisation of the cell membrane by limiting the penetration of surfactant into the lipid bilayer. The hydrophobic part would play the opposite role and promote the disruption of the membrane. Cremophor® El and Simulsol® 4000 have really similar structures and are characterised by the lowest IC50 among the studied surfactants. They are both synthesized from castor oil derivatives and conjugated to long PEG chains. Cremophor® EL was prepared by conjugation of ethylene oxide to castor oil at a molar ratio of 35:1 whereas Simulsol® 4000 contains 40 ethylene oxide units (PEG 40). In contrast, Polysorbate 80 and Solutol® HS15 have shorter PEGylated hydrophilic chains with approximately 20 and 15 units, respectively. Additionally, they differ by their hydrophobic part. Polysorbate 80 is a PEG sorbitane monooleate and Solutol® HS15 is mainly composed of PEG-hydroxystearate. Considering no significant size difference of hydrophobic parts, the low toxicity level of Simulsol® 4000 and Cremophor® EL would come from their hydrophilic PEG chains that is two times longer than the ones of Polysorbate 80 and Solutol® HS15. The well-known steric repulsion generated by long PEG chains could limit the penetration of surfactant into the lipid bilayer and consequently, preserve the cell integrity. One critical point is still the fact that some of the surfactants contain unbound PEG chains that do not contribute to the surface activity. Therefore, molar relationships between surfactant and their toxicity are of limited use and only mass related concentrations can at least basically reflect the comparative behaviour of the different surfactants.

It remains to be evaluated whether we deal here with a general phenomenon for fast degradable colloidal carriers, since mainly all require surfactants for their preparation, or it is only related to formulations were high concentrations of surfactants become the essential toxicity parameter.

5. Conclusion

LNC can be prepared from a variety of different non-ionic surfactants. Feasibility and stability were comparable in mainly all formulations. Generally, LNC show a strong surfactant type dependent toxicity in HaCaT cells. Differences were independent from their surface tension activity and only marginal correlations were found for chemical partial structures. The correlation of toxicity from pure surfactant and the respective LNC formulation suggests a limited stability of the LNC in its biological environment. Consequently, it seems to be possible to estimate the toxicological behaviour of LNC by evaluating the surfactant component alone.

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