European Journal of Pharmaceutics and Biopharmaceutics 76 (2010) 56–67

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Preservation of nanostructured lipid carriers (NLC)

Wasfy M. Obeidat [a], Kay Schwabe [b], Rainer H. Müller [b], Cornelia M. Keck [c,d,*]

[a] University of Sharjah, College of Pharmacy, United Arab Emirates
[b] Freie Universität Berlin, Department of Pharmaceutics, Biopharmaceutics & NutriCosmetics, Berlin, Germany
[c] Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia
[d] Fachhochschule Kaiserslautern, University of Applied Sciences, Pirmasens, Germany

ARTICLE INFO

Article history:
Received 23 February 2010
Accepted in revised form 3 May 2010
Available online 7 May 2010

Keywords:
Preservative
Nanostructured lipid carriers
NLC
Zeta potential
Physical stability

ABSTRACT

Due to their positive features (e.g., increased penetration of actives, re-enforcement of the lipid barrier and increase in skin hydration), nanostructured lipid carriers (NLC) are used in many dermal formulations. These formulations require preservation, and preservatives can impair the physical stability of disperse systems. Therefore, in this study, the influence of preservatives on the physical stability of Q10-loaded NLC was investigated using 11 different preservative mixtures. Whereas for nanosuspensions, only a limited number of preservatives are known from the literature not affecting their physical stability, a surprisingly high number of seven preservatives could be identified to be suitable for the preservation of NLC dispersions. For Q10-loaded NLC, Hydrolite 5 proved to be the best preservative, as it was found surprisingly to stabilize the NLC dispersion. Based on the data, a preservative classification system is suggested and a mechanistic model describing six key parameters affecting the physical stability of NLC could be developed. As most suitable characterization method to screen for suitable preservatives, light microscopy was identified. By being a simple, fast and cost efficient method, even extensive preservative screening studies can be performed very efficiently.

1. Introduction

Nanostructured lipid carriers (NLC) are solid lipid nanoparticles (SLN) of the second generation, which are used for the delivery of lipophilic actives. Advantages over conventional delivery systems are the protection of chemically labile compounds against chemical degradation and an increased bioactivity [1]. NLC can be administered, e.g., via the oral [2], ocular [3], pulmonary and the intravenous route [4]. However, at present, the most frequently applied administration route is dermal application, because upon the application of NLC on the skin, further positive aspects of the NLC can be exploited [5,6]. These effects are the re-enforcement of a damaged lipid film and the increase in skin hydration [7] which for example can be exploited in case of atopic eczema [8]. The increase in hydration further leads to an increased penetration of active, which can be exploited in pharma (e.g., dermal drug targeting [9]) but also in cosmetics, e.g., for coenzyme Q10 [10,11]. Nonetheless, the increase in skin hydration also leads to a reduction in wrinkle depth. Therefore, NLC can be considered an all over interesting carrier system for pharma, cosmetics, personal care but also nutrition [12]. The extraordinary pharmaceutical relevance of the lipid nanoparticles was recognized by the special issue devoted to them by Advanced Drug Delivery Reviews (ADDR) [13]. At present, more than 40 NLC products are available on the cosmetic market, and NLC for pharmaceutical and nutritional use are in development.

Product development involves ensuring the chemical, physical, but also microbial stability of the product during the shelf life. Therefore, if a product contains water and is not intended to be packed into a sterile single-unit packaging, the product needs to be preserved to avoid bacteria growth. NLC are produced using the hot high-pressure homogenization technique with homogenization temperatures of 80 °C and higher [14,15]. Due to these production conditions after the production, the NLC concentrate is sterile. Dermal formulations containing NLC are obtained by simply admixing an NLC concentrate (e.g., high lipid content, low water content) to the cream formulation. Most of the dermal products are fluid or semi-solid formulations, containing water in the outer phase and thus requiring preservation. Hence, even if a NLC dispersion could be stored without preservation, at least after admixing the NLC to the dermal formulation brings them into contact to preservatives. Due to safety reasons and due to the ease of storage and handling in most cases the preservation of the NLC concentrate is desired.

Preservatives are known that they can cause physical instability of disperse systems (e.g., emulsions, suspensions, liposomes, etc.) and therefore they can also impair the stability of NLC dispersions.

---

[*] Corresponding author at: Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia. Tel.: +49 (0) 6331 2483 31; fax: +49 (0) 6331 2483 44.
E-mail address: ck@ckc-berlin.de (C.M. Keck).

0939-6411/$ - see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2010.05.001

Physical instabilities of lipid nanoparticle dispersions range from particle growth due to aggregation, to gelling phenomena [16], previously described when using suboptimal surfactants for stabilization [17] or exposing SLN suspensions to strong light irradiation in stress tests or to high temperatures [18]. If NLC suffer from such physical instability, their special properties disappear. Therefore, physical stability must be ensured during the whole shelf life of the NLC formulations.

At present, no basic investigation about the influence of preservatives on the physical stability of NLC is available. Therefore, the aim of this study was to investigate the possible impairment of preservatives on the physical stability NLC systematically and to develop a powerful and effective preservative screening method by identifying suitable and redundant characterization methods.

1.1. Selection of preservatives

Preservatives being available are very different (e.g., way of antibacterial action, chemical structure, etc.) and the preservatives used are different for pharma, cosmetic and nutrition. In pharma, the preservatives used can be classified into five groups [19,20]:

1. Phenols and their derivatives (e.g., cresol and parabens).
2. Aliphatic and aromatic alcohols (e.g., ethanol, benzyl alcohol, phenoxyethanol).
3. Organic mercury compounds (e.g., thiomersalum).
4. Quaternary ammonium compounds (e.g., cetyl pyridinium chloride).
5. Carbon acids (e.g., sorbic acid and benzoic acid).

In cosmetics and personal care, the variety of preservatives is much broader. What types and which concentrations of preservatives are allowed to be used in the different products (e.g., rinse off or leave on products) is regulated e.g., in [21]. Preservatives can cause irritation on the skin and can lead to allergic reactions. As these effects are known to be concentration depended, the quantity of preservative should be as low as possible [22,23]. The quantity of a mono compound can be reduced by using mixtures of different preservatives which are known to act synergistically [24]. An example of such a synergistic mixture is Euxyl PE 9010. Euxyl PE9010 contains 90% phenoxyethanol and 10% ethylhexylglycerin (cf. Table 1). The addition of the latter reduces the amount of phenoxyethanol needed by up to 50%. Therefore, instead of mono compounds today in most cosmetic products, mixtures of preservatives are used to minimize the risk of contact dermatitis and skin irritation. The public awareness of possible side effects of preservatives is steadily increasing and therefore a new target in cosmetics and personal care is to use compounds possessing an antimicrobial activity, but simultaneously possessing another main skin-caring action. In this case, the formulation can be classified as preservative free, giving the formulation a clear advantage over others. Examples are Hydrolite 5 (pentylene glycol) which is mainly used as a skin moisturizer [25] and MultiEx Naturotics (cf. Table 1) which is mainly used as moisturizer and antioxidant.

In this study, 11 preservatives or mixtures of those were selected in respect to chemical differences (hydrophilic, more hydrophobic and very hydrophobic) and low irritation and allergy potential. Also, the new class of preservatives, which do not need to be declared as preservative due to their other skin-caring properties (e.g., Hydrolite 5 and MultiEx Naturotics), was included in this study. The concentration of preservatives required in a formulation not only depends on the type of preservative, but also on the degree of microbial exposure during the shelf live. Creams filled into a jar require a higher concentration then liquids sealed and opened ones for use. NLC concentrates are intermediate excipient to be used in the production process of creams and lotions and thus do not require high concentrations of preservative. Therefore, in this study, despite from the preservatives requiring definitely a certain concentration (e.g., ethanol 20%), concentrations were selected, being in the middle of the suggested concentration range. Table 1 gives an overview of the preservatives selected, their chemical composition, their concentration range typically used and the concentration selected for the study.

2. Materials and methods

2.1. Materials

NLC loaded with the antioxidant coenzyme Q10 (Q10) were used in this study. The composition of the basic NLC formulation was 14.45% Precifac® ATO (cetyl palmitate, kindly provided by Gattefossé, France), 0.75% Miglyol® (Caelo GmbH, Germany), 1.80% TegoCare® 450 (polyglyceryl-3-methylglucose distearate, Goldschmidt AG, Germany), 4.50% coenzyme Q10 (Q10, BIK Internationaler Handel GmbH, Switzerland) and ultra-purified water up to 100.0% (MilliQ, Millipore, USA). All concentrations are given in w/w%. The preservatives, source and concentrations used are shown in Table 1.

2.2. Methods

2.2.1. Production of Q10-loaded NLC

The Q10-loaded NLC dispersions were produced by the hot high-pressure homogenization method as described in the literature [26,27]. Briefly, the solid lipid (cetyl palmitate) was heated up to 85 °C with Miglyol® 812 and TegoCare® 450. Then, Q10 was dissolved in the lipid melt. The lipid phase was subsequently dispersed in water of the same temperature by a high-speed stirrer (UltraTurrax T 25, Janke & Kunkel GmbH, Germany) at 8000 rpm for 30 s. The obtained pre-emulsion was passed through a temperature-controlled high-pressure homogenizer (Micron LAB 40, APV Deutschland GmbH, Germany) applying two cycles at 800 bar and maintaining the homogenization tower temperature at 85 °C. The obtained hot o/w nanoemulsion was filled in silanized glass vials, which were immediately sealed. A water bath adjusted to 15 °C was used to control the cooling rate and NLC solidification.

2.2.2. Production of preserved NLC dispersion

The effective concentrations of the preservatives used were not the same. Therefore, the Q10-loaded NLC were produced as a concentrate with reduced water content (equal to the highest amount of preservative system to be added). The preservatives (and if necessary water up to 100.0%) were added to the NLC concentrate at room temperature by gentle stirring.

2.2.3. Physical short- and long-term stability of NLC

The samples were stored at room temperature for the period of one year in the dark. The particle size of the samples was measured using dynamic and static light-scattering techniques, as well as light microscopy. The surface charge of the particles was quantified by zeta potential measurements.

2.2.3.1. Particle size characterization. The mean particle size was analyzed using dynamic light scattering (DLS), also known as photon correlation spectroscopy (PCS), using a Malvern Zetasizer ZS (Malvern Instruments, Malvern, UK). PCS is a light-scattering technique that is used to obtain the hydrodynamic diameter (*z*-average, *z*-ave) and the polydispersity index (PI), which is a measure of the width of the particle size distribution [28–30]. Prior to the measurement, all samples were diluted using ultra-purified water to yield a suitable scattering intensity. Samples were analyzed at 25 °C using the general purpose mode.

Table 1
List of preservatives investigated, chemical composition, source, recommended concentration range and concentration used.

Name/trade name	Chemical composition	Source	Concentration in% (w/w)	
			Effective range	Used
Hydrolite® 5	Pentylene glycol (1,3-pentanediol)	COSNADERM Chemische Rohstoffe GmbH Ladenburg, Germany	1.5–5.0	3.25
Dermosoft® Octiol	Caprylyl glycol (octane-1,2-diol)	Dr. Straetmans Chemische Produkte GmbH Hamburg, Germany	0.5–1.0	0.75
Rokonsal® PB5	Phenoxyethanol 71–73%, methylparaben 14–15%, ethylparaben 5.5–6.1%, propylparaben 2.2–2.6%, butylparaben 3.4–3.8%, isobutylparaben 1.6–2%	ISP Biochema Schwaben GmbH Memmingen, Germany	0.3–1.2	0.75
Phenonip®	Phenoxyethanol 70–75%, methylparaben 14.5–16.5%, ethylparaben 3.3–4.3%, propylparaben 1.7–2.3%, butylparaben 3.4–4.3%, isobutylparaben 1.7–2.3%	Clairant UK Ltd.Leeds, UK	0.5–1.0	0.75
Euxyl® PE9010	Phenoxyethanol 90%, ethylhexylglycerin (1,2-propanediol, 3-(2-ethylhexyloxy) 10%	Schülke & Mayr GmbH Norderstedt, Germany	1.0	1.0
Propylene glycol	Propylene glycol, 1,2-propanediol	CG Chemikalien GmbH Laatzen, Germany	10.0	10.0
Ethanol 96%	Ethanol 96% (v/v)	CG Chemikalien GmbH Laatzen, Germany	>20v/v%	20.0
Euxyl® K700	Phenoxyethanol 30%, benzyl alcohol 30%, potassium sorbate 15%, tocopherol 15%, water 10%,	Schülke & Mayr GmbH Norderstedt, Germany	0.5–1.5	1.0
Euxyl® K702	Phenoxyethanol 77%, benzoic acid 13%, dehydroacetic acid 6%, polyaminopropylbiguanide 2%, ethylhexylglycerol 1%, water 2%	Schülke & Mayr GmbH Norderstedt, Germany	0.2–1.0	0.6
MultiEx Naturotics™	Magnolia Biondii Bark Extract 44%, Salix Alba (Willow) Bark Extract 15%, butylene Glycol 15%, Citrus Grandis (Grapefruit) Fruit Extract 6.0%, Camellia Sinensis Leaf Extract 6.0%, Propolis Extract 1.5%, Thujopsis Dolabrata Extract 0.15%, Chamomilla Recutita (Matricaria) Extract 0.16%, alcohol 0.1%, water 0.1%	MAWI Industrieagentur OHGLimburgerhof, Germany	1.0–3.0	2.0

To detect the possible presence of microparticles, the particle size of the formulations was also analyzed by static light scattering (SLS), known as laser diffractometry (LD), using a Malvern Mastersizer 2000 (Malvern instruments, Malvern, UK) with a measuring range from 20 nm to up to 2000 μm. The LD data were analyzed as volume distributions and are shown as median volume weighted diameters $d(v)$ 50%, $d(v)$ 90%, $d(v)$ 95% and $d(v)$ 99%. The diameter values indicate the percentages of particles possessing a diameter equal to or lower than the given value [31]. The results were analyzed using Mie theory with optical parameters 1.456 (real refractive index) and 0.01 (imaginary refractive index).

2.2.3.2. Light microscopy. SLS measurements can overlook larger particles besides a small-sized main particle population [32]. Therefore, light microscopy with and without polarized light should be used as an additional characterization method to detect possible large particles or to prove their absence without doubt [33,34]. The Q10-loaded NLC formulations were examined using an Orthoplan (Leitz, Germany) and an Olympus CX41 (Olympus Deutschland GmbH, Germany). Microscopic pictures were obtained from the undiluted NLC formulations at different magnifications [35].

2.2.3.3. Zeta potential measurements. The zeta potential (ZP), reflecting the electric charge on the particle surface and indicating the physical stability of a colloidal system, was measured by determining the electrophoretic mobility using the Malvern Zetasizer ZS (Malvern Instruments, UK). The measurements were performed in two different media (water and original dispersion medium). First, the ZP was assessed in purified water. To avoid fluctuations in the ZP due to variations in the conductivity of purified water which can range from 1 to 10 μS/cm, the conductivity of the water was adjusted to 50 μS/cm using 0.9% (w/w) NaCl solution. Secondly, the ZP was measured in the original dispersion medium of each sample. The original dispersion medium is a TegoCare® 450 solution with the preservative added to the sample. Samples were analyzed in triplicate at 25 °C. The measured electrophoretic mobility was converted into the zeta potential using the Helmholtz–Smoluchowski equation. As analysis mode the auto mode option was selected.

3. Results and discussion

3.1. Production of Q10-loaded NLC

High-pressure homogenization led to homogeneous and small-sized Q10-loaded NLC with a mean particle size of 196 nm and a PI of 0.12. The ZP in water was −46 mV and was −37 mV in the original dispersion medium (Tegocare 450 solution). The high zeta potential in water indicates a high stern potential and thus a good steric repulsion of the NLC particles. The increase in the zeta potential when measured in water and compared to the ZP measured in the original dispersion medium is attributed to the desorption of some non-ionic stabilizer molecules from the surface of the particles, causing a shift of the plane of shear toward the particle surface [36,37].

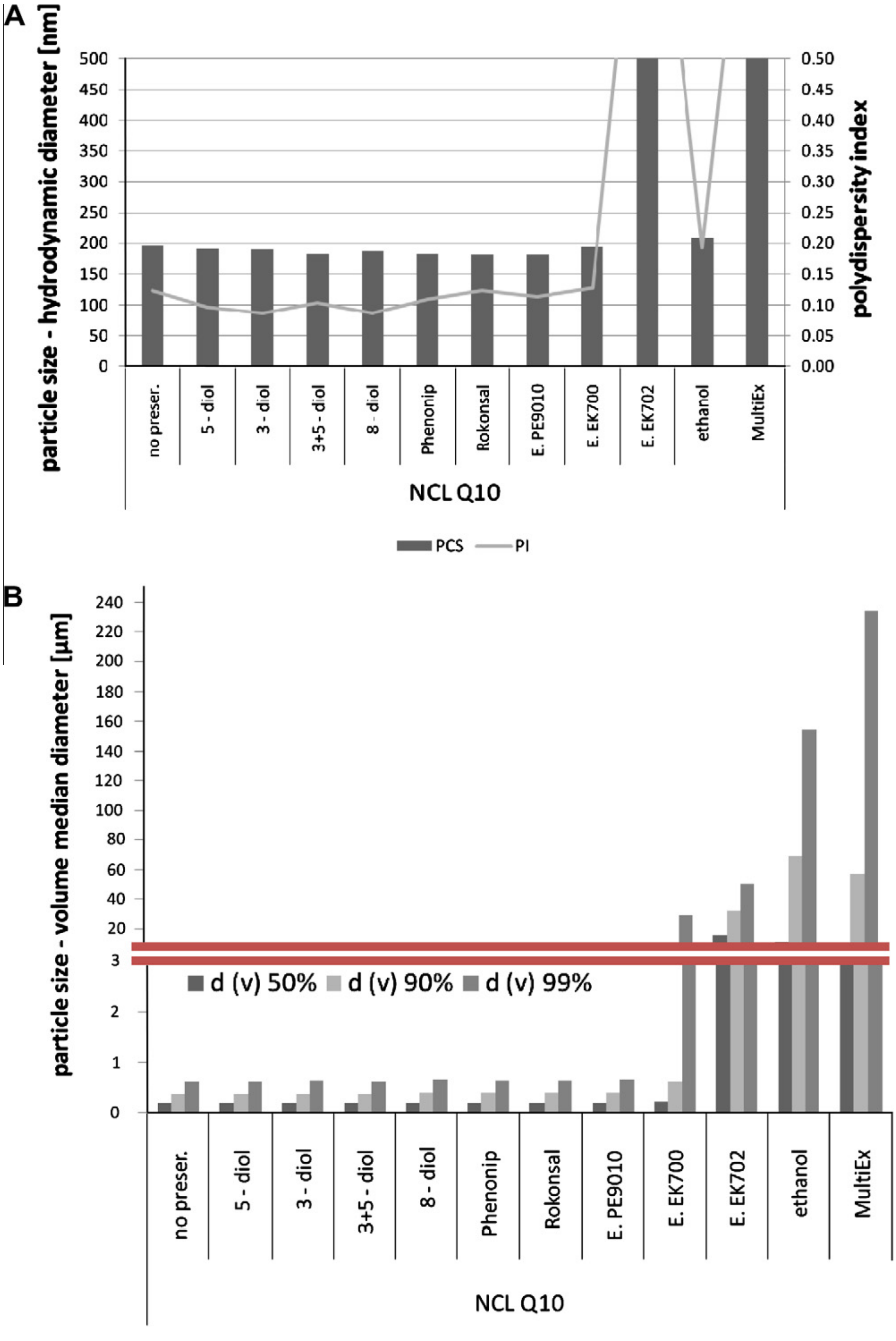

Fig. 1. Immediate influence of preservatives on the physical stability of Q10-loaded NLC: (A) PCS data and (B) LD data, diameters *d*(*v*) 50–*d*(*v*) 99%. *Abbreviations used: 5-diol = pentylene glycol, 3-diol = propylene glycol, 3 + 5-diol = mixture of pentylene and propylene glycol, 8-diol = caprylyl glycol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Production of preserved NLC dispersions

The preservatives selected were added to the NLC dispersion and the particle size was assessed within 24 h after the addition. The results of the PCS and LD measurements are shown in Fig. 1.

DLS measures the mean particle size and does not detect particles >6 µm. From this set of data, only two preservatives (Euxyl K702 and MultiEx Naturotics) impair the stability of the NLC dispersion. All other systems do not impair the size and the PI of the original NLC dispersion (Fig. 1A). LD measures larger particles to up to 2 mm. The diameters *d*(*v*) 90% and 99% are used to estimate the presence and the quantity of larger particles in the system, whereas the median *d*(*v*) 50% is a measure for the mean particle size of the system. From these data, not only Euxyl K702 and MultiEx Naturotics impair the physical stability of the NLC dispersion, but also Euxyl K700 and ethanol (Fig. 1B). However, the degree of aggregation is little for Euxyl K700, as only the *d*(*v*) 99% is large, whereas *d*(*v*) 50% and *d*(*v*) 90% are small. The other three preservatives cause strong aggregation (large *d*(*v*) 50% and *d*(*v*) 90%).

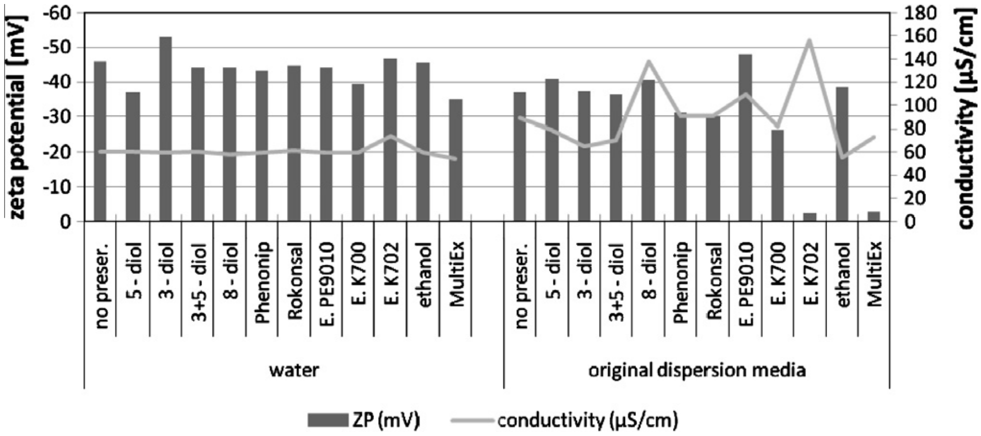

Fig. 2. Zeta potential of non-preserved NLC and preserved NLC measured in conductivity water (50 µS/cm, left) and in the original dispersion media (right).

3.2.1. Zeta potential measurements

The zeta potentials were measured in water and in the original dispersion medium. In order to obtain the original dispersion medium, Tego Care 450 was dissolved in hot water to yield a clear solution. However, during cooling, some Tego Care precipitated, because of its high lipophilic character (HLB value 12). The contribution of the precipitate to the scattering intensity was analyzed and was negligible. Therefore, the measured zeta potential originates primarily from the NLC systems investigated. The results obtained are shown in Fig. 2.

No significant changes in ZP were detected for most of the preservatives, when the ZP values were assessed in water. The differences were not larger than 3 mV. For four preservatives, the changes were larger. Upon the addition of pentylene glycol (5-diol), the ZP decreased from −46 mV to −37 mV, for Euxyl K700 the ZP decreased to −39 mV and in case of MultiEx Naturotics it decreased by 11 mV down to −35 mV (Fig. 2 left). In case of propylene glycol, the ZP increased by 7 mV to −53 mV.

More differences in the ZP were found when measurements were conducted in the original dispersion media (Fig. 2 right). No significant changes in the ZP were found for all glycols (propylene glycol (3-diol), pentylene glycol (5-diol), mixture of them (3 + 5-diol) and caprylyl glycol (8-diol) and for ethanol. A small decrease in ZP (6–7 mV) was obtained for the preservatives containing parabens (Phenonip and Rokonsal PB5). Euxyl K700 decreased the ZP by 11 mV, whereas it was increased by 11 mV for Euxyl PE9010. A tremendous decrease in the ZP by 34 and 35 mV was obtained for the most destabilizing preservatives MultiEx Naturotics and Euxyl K702. The effects observed can be explained by the zeta potential theory.

In water, the diffuse layer is very thick (about 300 nm), and the decay of the potential in this diffuse layer is very slow [36]. Therefore, when measured in water, the zeta potentials (measured potentials at the plane of shear) are practically identical to the Stern potential. Thus, in practise, the measured zeta potential (when analyzed in water!) is set equal to the Stern potential. The Stern potential itself is directly related to the Nernst potential, which is the surface potential and thus a measure of the particle surface charge (e.g., caused by dissociation of functional groups, adsorbed ions present in the distilled water). Hence, by diluting a sample in water prior to the measurement reveals the Stern potential. In this study, the same NLC were used for all preserved samples. Therefore in theory, independent on the added preservative, in water, a similar Stern potential should be measured for all samples. This was the case for most of the NLC (Fig. 2). However, deviations can occur if molecules (e.g., preservatives) are tightly adsorbed on the particle surface and do not desorb upon the dilution with water. In this study, this was especially the case for MultiEx and for pentylene glycol. However, all measured potentials are above I30I mV, thus all particles would have a sufficient long-term stability in the environment of water.

This is different, when the environment is the original dispersion medium. Measuring the ZP in the original dispersion medium is a measure of the thickness of the diffuse layer [36]. The lower the zeta potential, the thinner the diffuse layer and the less stable is the particle dispersion. According to Riddick, a ZP of larger than I30I mV is required for a long-term stable dispersion [38]. This was found for the non-preserved NLC dispersion, and for the seven stable preserved NLC dispersions (Fig. 2, right). In contrast to this, the ZP values measured for the NLC dispersion being preserved with Euxyl K700 and MultiEx Naturotics were close to zero (2.4, 2.9 mV). Therefore the observed instability (cf. 3.2 and Fig. 1B) can be explained by the ZP theory. Euxyl K700 exhibited a higher zeta potential of −26.4 mV, which indicates instability but slower aggregation, which was fully confirmed by the PCS and LD data (e.g., no increase in PCS diameter, only a slight increase in $d(v)$ 99% and no increase in $d(v)$ 50% and $d(v)$ 90% one day after addition of the preservative (cf. Fig. 1B).

Nevertheless, the ZP is not the only stability determining parameter. Preservatives adsorb to a certain extent onto the surface, interact with the stabilizer layer and thus can destabilize a system [39]. Such an interaction is not necessarily reflected in a change of the zeta potential. An example for such an interaction is the NLC dispersion preserved with ethanol. The zeta potential is −38.6 mV, hence from ZP theory, the dispersion is in the range of ZP values for stable dispersions. Within this study, however, aggregation was found (cf. Fig. 1B). Therefore, the instability must be attributed to destabilizing effects not accessible by a ZP measurement. It is known that ethanol can compete with the stabilizing surfactant for hydration water. In a high concentration of 20% ethanol used in this study, this can lead to a partial dehydration of the surfactant, which subsequently affects the NLC stability.

3.2.2. pH-values

The pH of the unpreserved NLC dispersion was 6.88 and only very small changes (±0.3) were obtained upon the addition of the preservatives. One exception was Euxyl K702 which led to a pH value of 4.17. The pH is very important for the physical stability of colloidal systems, as it strongly influences the zeta potential [36,37]. Hence, changes in the pH can cause instability (i.e., agglomeration) of a system due to a decrease in the zeta potential. Therefore, it remains unclear whether the instability caused by the

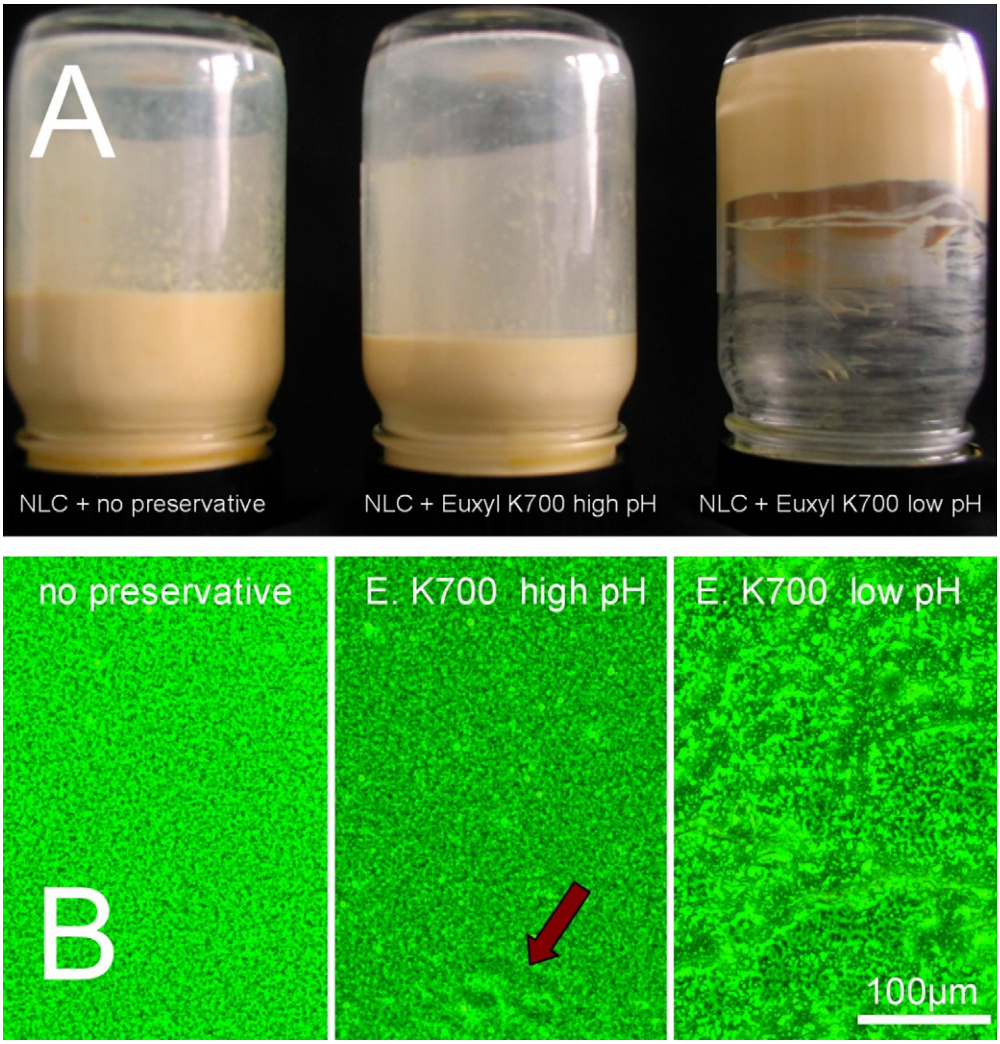

Fig. 3. Influence of pH on physical stability of NLC: macroscopic images (upper), microscopic images (lower), explanation cf. text (arrow = slight aggregation). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

addition of Euxyl K702 is due the chemical nature of the preservative or due to the drop in pH. On the other hand, the pH is also important for the antimicrobial action of many preservatives. One example is sorbic acid (IUPAC name: 2,4-hexadienoic acid). Only the protonated acid acts as preservative, whereas its deprotonated form sorbate does not. Thus, sorbic acid with a p_{ka} value of about 5 must be used in formulations with low pH only; otherwise contamination with microorganisms cannot be prevented. Euxyl K700 contains potassium sorbate (cf. Table 1) and the pH of the system must be below 5.5 [40]. The NLC dispersion preserved with Euxyl K700 only showed a slight impairment which was not detected by PCS measurements, but by a high $d(v)$ 99% value (cf. 3.2 and Fig. 1). However, the pH of the preserved NLC dispersion was 7.05; hence no antimicrobial action was achieved.

To investigate the influence of the pH on the impairment of NLC, the sample containing Euxyl K700 was divided into two parts. One part was kept at the original pH, and the other part was adjusted to the required pH of 5.5 using citric acid (10% w/w). The samples were investigated macroscopically and by light microscopy and compared to the NLC without preservative. The results are shown in Fig. 3. Macroscopically, no differences are visible between the systems containing no preservative and Euxyl K700 at high pH (Fig. 3A left and middle). High instability was found when the system was adjusted to a pH of 5.5. The NLC agglomerated, leading to complete solidification of the whole system (Fig. 3A right). Also, light microscopy confirms the strong pH dependency on the stability. No aggregation was found for the NLC without preservative (Fig. 3B left), and only a slight aggregation was obtained for the sample with Euxyl K700 at high pH (indicated by arrow, Fig. 3B middle). However, strong aggregation was observed for the sample with low pH (Fig. 3B right).

3.3. Physical short- and long-term stability of NLC

3.3.1. Physical short-term stability

After the production, the samples were stored at room temperature and analyzed for their size after 1 week (d7) and 1 month (d30) of storage. The results are shown in Fig. 4.

From PCS measurements (Fig. 4A), a small increase in size was only observed for ethanol. All other systems remained unchanged when compared to day 1 (cf. 3.2). Also, LD results remained almost unchanged over this time of observation (Fig. 4B). All systems that remained unchanged immediately after the addition of preservative remained small sized, whereas the aggregated systems Euxyl K700, K702, ethanol and MultiEx Naturotics remained large in size.

3.3.2. Physical long-term stability

All samples were further stored at room temperature and characterized after 3, 6 and 12 months of storage. The data obtained are shown in Fig. 5.

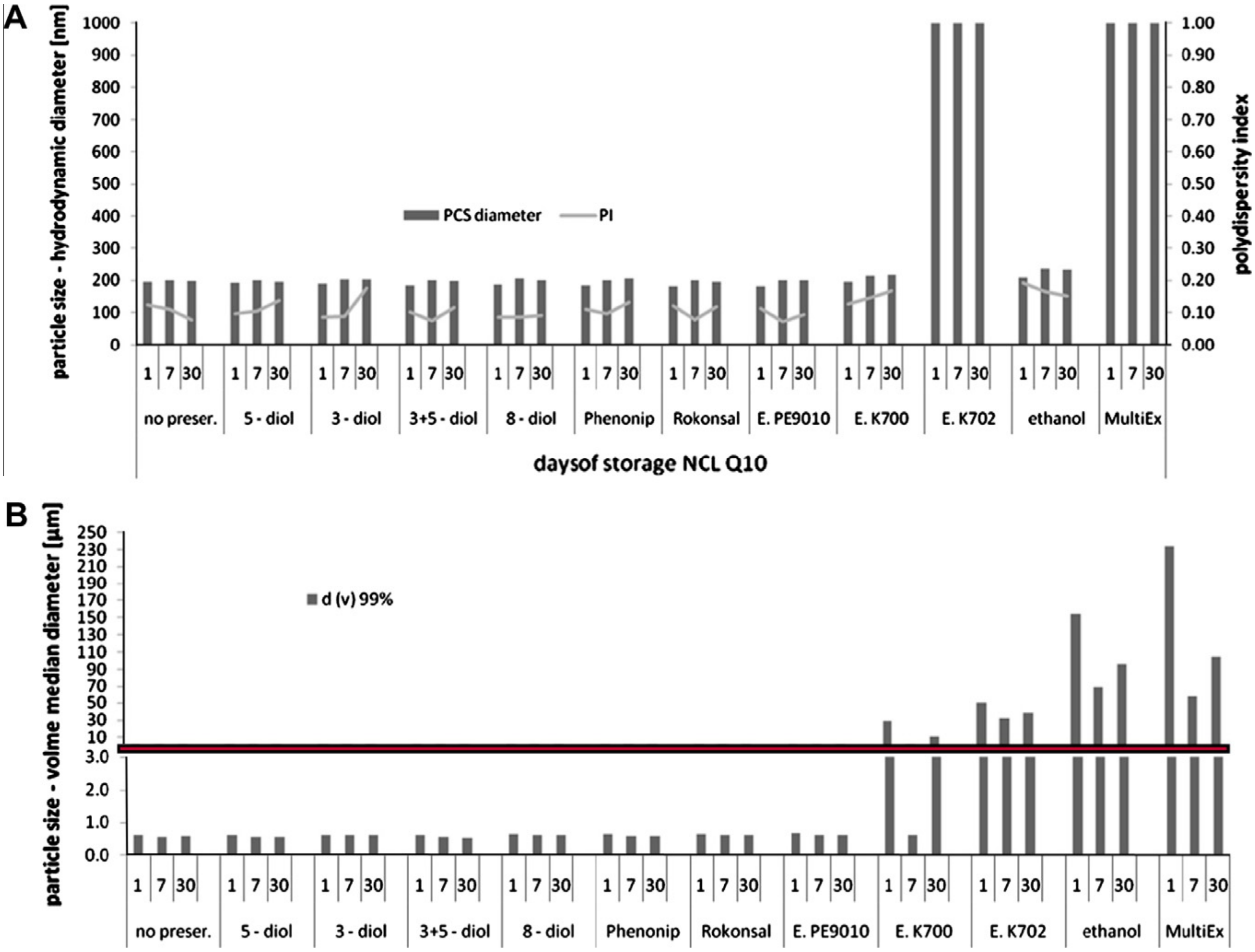

Fig. 4. Influence of preservatives on the physical short-term stability (1 month) of Q10-loaded NLC: (A) PCS data (hydrodynamic diameters and polydispersity indices – PI) and (B) LD data (diameters $d(v)$ 99% plotted as function of time (day 1, 7, 30)).

PCS data (Fig. 5A) again led to no changes for the non-preserved NLC and for the systems preserved with propylene glycol, pentylene glycol, the mixture of these preservatives, Euxyl 9010 and Rokonsal PB5. For the NLC preserved with Phenonip, a small increase in size was obtained over the one-year storage. However, a pronounced increase was obtained for the instable Euxyl K700 and ethanol systems as seen previously by LD data (cf. 3.2). The data obtained by LD (Fig. 5B) show that there is certain instability of the non-preserved NLC concentrate after 6 months of storage, which can be seen by a slight increase in the $d(v)$ 95%. The instability was more pronounced after 12 months of storage. After 12 months of storage, also the $d(v)$ 90% increased and the $d(v)$ 95% further increased. However, the degree of aggregation is still low and the aggregates are loose. This can be estimated by a repeated measurement, where the stirrer within the instrument de-aggregates the larger particles (meas. 2) leading to a smaller size. By further comparing the LD data, it becomes obvious that after 6–12 months of storage, a further differentiation between the different preservatives is possible. The NLC preserved with caprylyl glycol (8-diol) and Phenonip are slightly less stable than the original non-preserved NLC concentrate, which can be seen by a higher particle size but also by the differences in the repeated measurement, indicating a stronger degree of agglomeration. No impairment or even a slight stabilization effect was observed for the NLC systems preserved with Rokonsal PB5, Euxyl PE9010 and propylene glycol. A clear stabilizing effect was obtained for the systems preserved with pentylene glycol and the mixture of pentylene and propylene glycol.

3.4. Mechanistic consideration

In this study, eleven different systems of preservatives were investigated regarding their influence on size, physical stability and zeta potential of a NLC dispersion. Six of these preservatives were previously employed for the preservation of a Hesperetin nanosuspension (i.e., drug nanocrystals dispersed in water) [39]. In this previous study, all preservatives used impaired the stability of the nanosuspension. However, it was possible to differentiate them into the group of least impairment (1: pentylene glycol, 2: Euxyl PE9010, 3: Rokonsal PB, 5 and 4: Phenonip) and into the group of pronounced impairment (caprylyl glycol and MultiEx Naturotics). In the present study, some additional preservatives and mixtures were used to provide a broader data base for a better mechanistic understanding of the interaction between preservatives and NLC. In this present study, now a differentiation is not only possible into two groups, but into four classes (Table 2).

The strong destabilizing effect (class III preservatives) was found to be an immediate effect that occurs and can be detected very shortly upon the addition of the preservative. The differentiation between class I, II and IV is a long-term effect, which can be discriminated only after a long time of storage (e.g., after

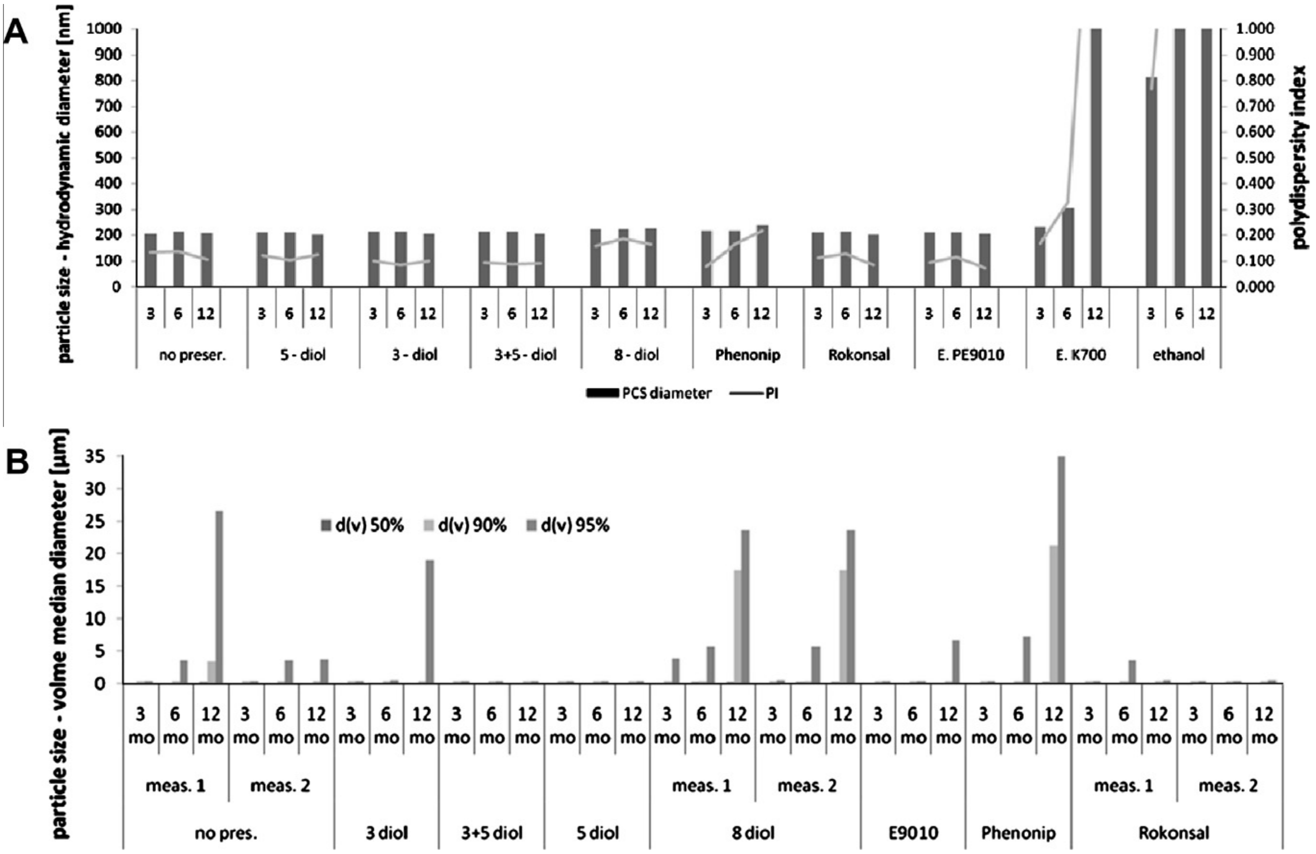

Fig. 5. influence of preservatives on the physical long-term stability (1 year) of Q10-loaded NLC: (A) PCS data (hydrodynamic diameters and polydispersity indices – PI) and (B) LD data (diameters $d(v)$ 50–$d(v)$ 95% plotted as function of time (1 month, 6 months, 1 year)).

Table 2
The four classes of the classification system of preservatives (CSP) and corresponding preservatives identified in this study.

Class	Kind of impairment	Preservatives belonging to the group
I	No stability impairment	• Propylene glycol • Rokonsal PB5 • Euxyl PE9010
II	Little stability impairment	• Caprylyl glycol • Phenonip
III	Strong impairment of stability	• Euxyl K700 • Euxyl K702 • MultiEx Naturotics • Ethanol
IV	Stabilizing effect	• Pentylene glycol • Mixture of pentylene and propylene glycol

6–12 months). The preservatives affected the stability of the NLC differently than the stability of the nanocrystals. For example:

- Pentylene glycol showed least interference for the nanocrystals, but possessed a stabilizing effect when NLC were preserved with it.
- Caprylyl glycol which was destabilizing the nanocrystals in a pronounced way, affected the stability of the NLC dispersions only little.
- No difference in impairment was found for Phenonip an Rokonsal for the nanosuspensions, but for NLC Phenonip proved to impair the stability of NLC more than Rokonsal PB 5 did.
- MultiEx Naturotics destabilized both nanocrystals and lipid nanoparticles.

One difference between the two studies is the type of stabilizer used. The nanosuspensions were stabilized with PlantaCare 2000 (alkyl (8-16) glucoside) whereas TegoCare 450 (polyglyceryl-3-methyl glucose distearate) was used for the NLC. However, both stabilizers are non-ionic, thus the general interaction with the preservatives should be rather similar and the differences should be related to other aspects. The physical stability of colloidal systems or the effect of destabilization is actually a multifactorial phenomenon, depending on many parameters, e.g., on:

1. affinity of the preservative to the particle surface,
2. surface hydrophobicity of the particles,
3. anchoring of stabilizer onto/into surface,
4. ability of preservative to reduce zeta potential,
5. nature of the particle stabilizer, and
6. interaction of preservative with stabilizer layer.

The influencing parameters listed from above are depicted in Figs. 6 and 7 to explain these effects in more detail. Fig. 6A explains the influence of the affinity of the preservative and the effect of surface hydrophobicity (parameters 1 and 2). Preservatives vary in their lipophilicity and can be classified into hydrophilic (e.g., propylene glycol), hydrophobic (e.g., caprylyl glycol, parabens) and highly lipophilic (e.g., MultiEx). Particles dispersed within an aqueous system can be classified into hydrophobic (e.g., Hesperetin nanocrystals) and very lipophilic (e.g., Q10 NLC). Here, the classification hydrophilic does not exist, as these particles would dissolve within the water phase. Upon the addition of the preservatives to the particles, the impairment in stability will depend on the combination of class of preservative/class of particle. Hydrophilic preservatives (e.g., propylene glycol) have no tendency to

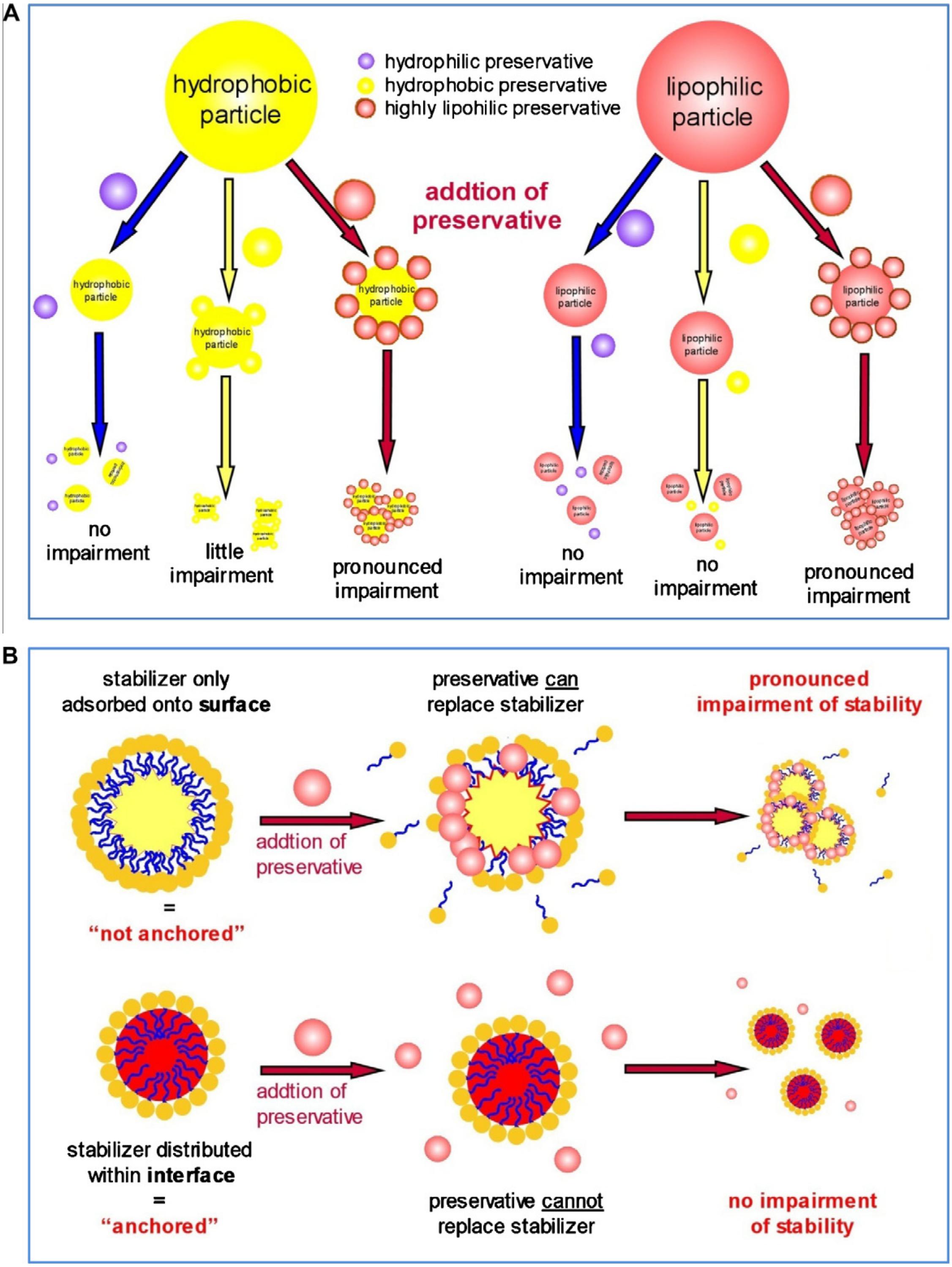

Fig. 6. Parameters playing a key role in the stability of colloidal systems and mechanism of impairment due to the addition of preservatives. (A) Influence of affinity of the preservative to the particle surface and influence of surface hydrophobicity of the particles and (B) influence of anchoring of stabilizer onto/into surface (explanations cf. text). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

adsorb to the surfaces of the particles. More hydrophobic preservatives (e.g., caprylyl glycol) adsorb onto the hydrophobic surface of nanocrystals affecting their stability but are not lipophilic enough to adsorb onto the lipophilic surface of the NLC. Therefore, NLC are not impaired by these preservatives. Lipophilic compounds (e.g., Honokiol and Magnolol, compounds of the preservative MultiEx Naturotics, cf. Table 1) adsorb to all surfaces and thus impair the stability of both the nanocrystals and the NLC.

Fig. 6B shows the influence of the anchoring of the stabilizer onto/into the particle surface (parameter 3). In case of nanocrystals, the stabilizer is not anchored within the interface, but is only adsorbed onto the surface; therefore, it can be desorbed from the

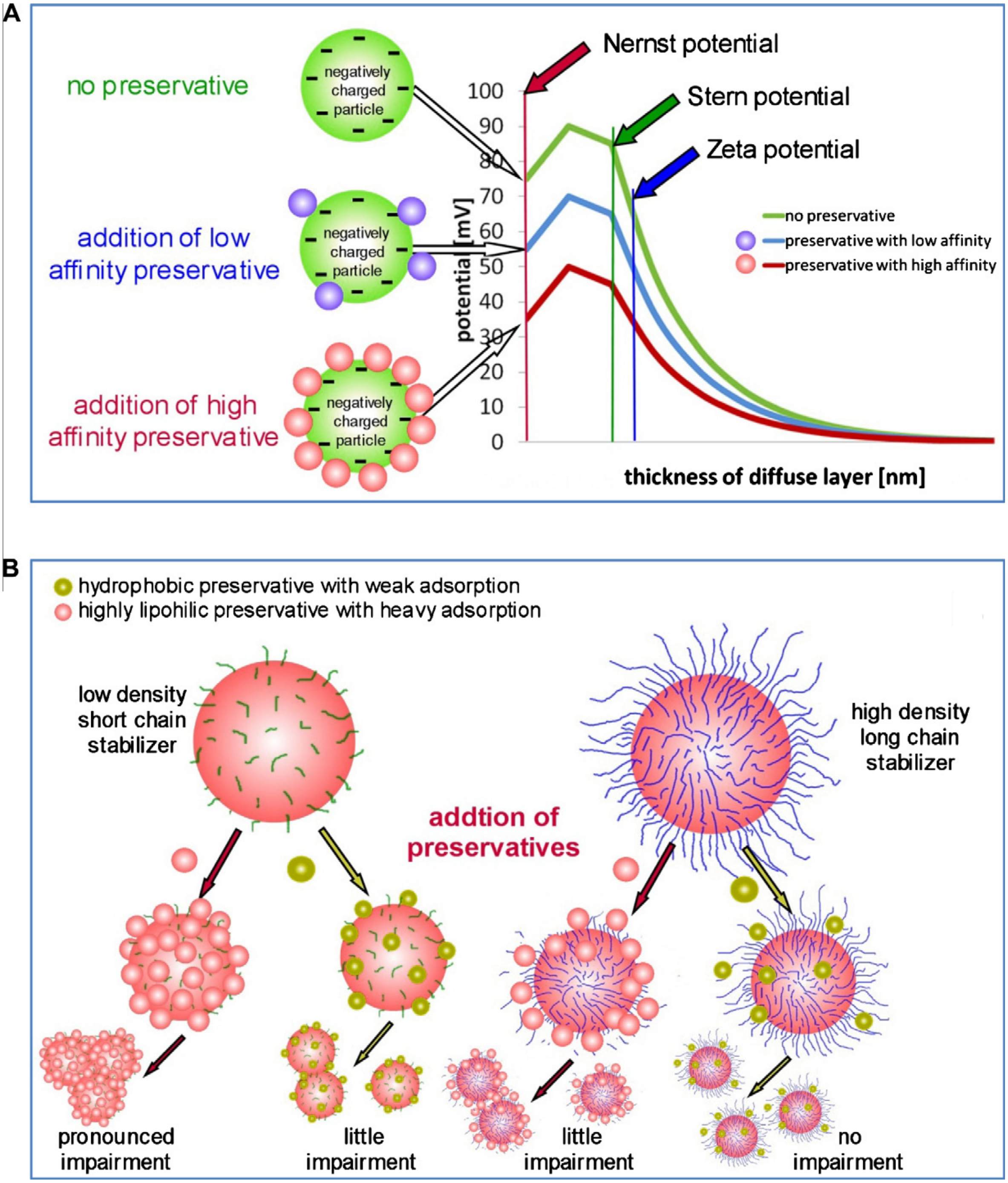

Fig. 7. Parameters playing a key role in the stability of colloidal systems and mechanism of impairment due to the addition of preservatives. (A) Capability of preservative to reduce the zeta potential and (B) influence of nature of the particle stabilizer and influence of interaction of preservative with stabilizer layer (explanations cf. text). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surface easily and can be replaced by the adsorbing preservative easily (Fig. 6B upper). In contrast, many stabilizers of emulsions, SLN and NLC are really anchored within the interface. There is little chance to desorb these surfactants, especially in the case of solid nanoparticles, and thus no impairment is expected (Fig. 6B lower).

The capability of a preservative to reduce the zeta potential is explained in Fig. 7A. Due to the absorbance of preservative, charges of the surface of the particle can be covered. This leads to a reduction in the Nernst potential and subsequently to the reduction in Stern and zeta potential. In case of low affinity to the surface, the reduction is less pronounced (e.g., Euxyl K700, Phenonip and Rokonsal PB5). If the affinity of the preservative is high, the coverage is pronounced and the reduction in the ZP is strong (e.g., close to zero, i.e., MultiEx Naturotics). Finally, the interaction of preservative with the stabilizer layer and the influence of the nature of stabilizer (parameters 5 and 6) are explained in Fig. 7B. Properties, e.g., packing density of the surfactants on a particle surface, stabilizer layer thickness and the degree of intermolecular forces (=determining the rigidity of the stabilizer film) depend on the nature of stabilizer used and strongly influence the stability of a colloidal system. Preservatives can adsorb and localize themselves in between the stabilizer molecules. Similar to the interactions between the particle surface and the preservative (cf. Fig. 6A), the degree of interaction between the stabilizer and the preservative will depend on the nature of stabilizer and preservative (e.g., can lead to no or little weakening of the molecular interaction and packing density, or a strong molecular interaction between the preservative and stabilizer molecules). To which extent the stability of the colloidal system will be impaired will depend on whether or not the destabilization effect of the preservative can be compensated by the stabilizer film. If the packing density is low, and the steric effect limited (too short sterically stabilizing chains, too thin stabilizer layer), the particles will aggregate (Fig. 7B left). In case of a dense packing and long tails (i.e., thick adsorbed layer), the stabilizer will be able to compensate the destabilization by the preservative (Fig. 7B right). Ideally, the thickness of the adsorbed layer should be above 10 nm for complete steric stabilization [41]. However, as there is an overlapping of destabilizing effects of the preservative and stabilizing and potentially compensating effects by the stabilizer, it is very difficult to predict the degree of impairment. For example, in case of pentylene glycol and MultiEx: for both a reduction in ZP was analyzed in water (cf. Fig. 3), indicating the adsorption of the preservative onto the surface of the particles. Whereas MultiEx strongly impaired the stability of the NLC, pentylene glycol even increased the stability. Another example is Rokonsal PB5 and Phenonip, the composition of the preservatives is almost identical (cf. Table 1) but the degree of impairment was different (cf. Fig. 5B). The only difference is the content of longer alkyl chain parabens (e.g., butyl- and isobutylparaben) in case of Phenonip. Longer alkyl chains posses a higher lipophilicity explaining the increased instability of NLC containing Phenonip in comparison with NLC containing Rokonsal PB5.

3.5. Analytical consideration

The characterization of the systems was performed using photon correlation spectroscopy, laser diffraction, zeta potential measurements and light microscopy. Besides light microscopy, which is simple, low cost and easy to perform, all other techniques applied require some knowledge and experience to obtain reliable results [33]. In addition, these techniques are expensive and not available in every laboratory. Therefore, it is also important to judge, if the use of these techniques is useful and really needed or if the same reliable result can be obtained using simple techniques. The aim of this study was to investigate the mechanism of impairment due to the addition preservatives. However, in product development, it is only important to screen for preservatives which do not influence the physical stability of the colloidal system. Instability is characterized by aggregation of the nanoparticles, leading to the formation of larger particles, which become visible under a light microscope. PCS is applied to measure the mean particle size of nanoparticles; it is sensitive to small changes and thus a good, early indicator for physical instability. Nevertheless, in this study, only the preservatives leading to pronounced and immediate instability could be discriminated from the other groups by PCS, no differentiation was possible for the other three groups. Zeta potential measurements are normally performed in water. The results obtained for the samples investigated did not explain the instability observed. Only LD measurements yielded clear results and enabled the differentiation between the four groups of preservatives. However, all LD results (e.g., no impairment, slight aggregation, heavy aggregation) could be fully confirmed by light microscopy. Therefore, it can be concluded that light microscopy alone can be used to screen for the best preservative in formulation development.

4. Conclusion

Q10-loaded NLC were produced by hot high-pressure homogenization. The addition of different preservatives impaired the physical stability of the original NLC dispersion without preservatives. Based on the data, a preservation classification system was developed, to enable a fast differentiation of the different preservatives. Class III preservatives (pronounced impairment) can be identified immediately, whereas the differentiation between class I (no impairment), II (slight impairment) and VI (stabilizing effect) is a long-term effect. The degree of impairment is an interplay of many parameters, where already small differences (e.g., changes in pH and almost negligible differences in the preservative composition (e.g., Rokonsal and Phenonip) can lead to great differences in the dispersion stability. As basic rules for achieving stable preserved nanodispersions, the preservative should be as hydrophilic as possible with little affinity to the particle surface and ideally non-ionic to minimize zeta potential reduction. The stabilizer should be bound as firm as possible to the surface, ideally being anchored into the particle matrix (as in NLC). Stabilizers used should be able to compensate as much as possible the destabilization effect caused by the preservative (e.g., stabilizer should adsorbed in a dense packaging and should have a thick sterically stabilizing layer of long polymer tails). Based on this model developed, an ideal preservative cannot yet be predicted by a computer program, but the parameters identified can be used as helpful guidelines in the development of preserved nanodispersions. The most effective screening (reliable, fast and cost effective) can be performed using a simple light microscope.

Acknowledgements

The authors would like to thank the DFG (Germany) and JUST (Jordan) for providing the support for this study. Materials and equipment were provided by PharmaSol GmbH Berlin. The Authors thank Ms. Corinna Schmidt for her assistance in the particle size measurements.

References

[1] R.H. Müller, M. Radtke, S.A. Wissing, Nanostructured lipid matrices for improved microencapsulation of drugs, Int. J. Pharm. 242 (1–2) (2002) 121–128.
[2] M. Muchow, P. Maincent, R.H. Müller, Lipid nanoparticles with a solid matrix (SLN, NLC, LDC) for oral drug delivery, Drug Dev. Ind. Pharm. 34 (12) (2008) 1394–1405.
[3] R. Cavalli, M.R. Gasco, P. Chetoni, S. Burgalassi, M.F. Saettone, Solid lipid nanoparticles (SLN) as ocular delivery system for tobramycin, Int. J. Pharm. 238 (1-2) (2002) 241–245.
[4] M.D. Joshi, R.H. Müller, Lipid nanoparticles for parenteral delivery of actives, Eur. J. Pharm. Biopharm. 71 (2) (2009) 161–172.
[5] R.H. Müller, R.D. Petersen, A. Hommoss, J. Pardeike, Nanostructured lipid carriers (NLC) in cosmetic dermal products, Adv. Drug Deliv. Rev. 59 (6) (2007) 522–530.
[6] J. Pardeike, A. Hommoss, R.H. Müller, Lipid nanoparticles (SLN, NLC) in cosmetic and pharmaceutical dermal products, Int. J. Pharm. 366 (1-2) (2009) 170–184.
[7] S.A. Wissing, R.H. Müller, The influence of solid lipid nanoparticles on skin hydration and viscoelasticity – in vivo study, Eur. J. Pharm. Biopharm. 56 (1) (2003) 67–72.
[8] C.M. Keck, K. Schwabe, Silver–nanolipid complex for application to atopic dermatitis skin: rheological characterization, in vivo efficiency and theory of action, J. Biomed. Nanotechnol. 5 (4) (2009) 428–436.
[9] C. Santos Maia, W. Mehnert, M. Schaller, H.C. Korting, A. Gysler, A. Haberland, M. Schäfer-Korting, Drug targeting by solid lipid nanoparticles for dermal use, J. Drug Target. 10 (6) (2002) 489–495.
[10] J. Pardeike, R.H. Müller, Coenzyme Q10-loaded NLCs: preparation, occlusive properties and penetration enhancement, Pharm. Technol. Eur. (01), July 2007 (online journal).
[11] J. Pardeike, K.H. Schulz, T. Miller, R.H. Müller, Coenzyme Q10 loaded NLC: in vitro release and in vivo skin penetration, in: AAPS Annual Meeting & Exposition, San Diego, CA, 2007.
[12] R.H. Müller, C.M. Keck, Nanostructured lipid carriers (NLC) for the use in pharma, cosmetics, food and agri. particles, Orlando, 2004.
[13] C.J.H. Porter, K.M. Wasan, P. Constantinides, Lipid-based systems for the enhanced delivery of poorly water soluble drugs, Adv. Drug Deliv. Rev. 60 (6) (2008) 615–778.
[14] J.S. Lucks, R.H. Müller, Medication vehicles made of solid lipid particle (solid lipid Nanospheres-SLN), European Patent EP19920919477 1992, MEDAC GmbH.
[15] R.H. Müller, V. Jenning, K. Mäder, A. Lippacher, Lipid particles on the basis of mixtures of liquid and solid lipids and the method for producing same, PCT/

EP2000/004112, European Patent Application EP1176949, PharmaSol GmbH, 2000.
[16] A. Lippacher, R.H. Müller, K. Mäder, Semisolid SLN dispersions for topical application: influence of formulation and production parameters on viscoelastic properties, Eur. J. Pharm. Biopharm. 53 (2) (2002) 155–160.
[17] K. Westesen, B. Siekmann, Investigation of the gel formation of phospholipid-stabilized solid lipid nanoparticles, Int. J. Pharm. 151 (1997) 35–45.
[18] C. Freitas, R.H. Müller, Correlation between long-term stability of solid lipid nanoparticles (SLN) and crystallinity of the lipid phase, Eur. J. Pharm. Biopharm. 47 (2) (1999) 125–132.
[19] K.H. Bauer, K.-H. Frömming, C. Führer, Lehrbuch der Pharmazeutischen Technologie, Wissenschaftliche Verlagsgesellschaft mbH, Suttgart, 2002.
[20] R. Voigt, Pharmazeutische Technologie, Deutscher Apotheker Verlag, Stuttgart, 2006.
[21] Kosmetik-Verordnung in der Fassung der Bekanntmachung vom 7, Oktober 1997 (BGBl. I S.2410), die zuletzt durch die Verordnung vom 3, Februar 2010 (BGBl. I S. 65) geändert worden ist.
[22] W. Umbach, Kosmetik und Hygiene: von Kopf bis Fuß, Wiley VHC, Weinheim, 2004.
[23] Mekos Laboratories, PRODUCT INFORMATION – Allergen Patch Test T.R.U.E. TEST, 2007.
[24] Schülke & Mayr GmbH, Euxyl® PE 9010, Product information, Preservatives for cosmetics <http://www.schuelke-mayr.com>.
[25] A.G. Symrise, Hydrolite® 5 – product information, Active Ingredients <http://www.symrise.com>.
[26] W. Mehnert, K. Mäder, Solid lipid nanoparticles: production, characterization and applications, Adv. Drug Deliv. Rev. 47 (2–3) (2001) 165–196.
[27] S. Liedtke, S. Wissing, R.H. Muller, K. Mader, Influence of high pressure homogenisation equipment on nanodispersions characteristics, Int. J. Pharm. 196 (2) (2000) 183–185.
[28] R.H. Müller, R. Schuhmann, Teilchengrößenmessung in der Laborpraxis, Band 38, in: A. Paperback (ed.), Wissenschaftliche Verlagsgesellschaft mbH, Suttgart, 1996.
[29] Malvern, Dynamic Light Scattering: An Introduction in 30 min, DLS technical note: MRK 656-01.
[30] International Organisation for Standardisation (ISO), International Standard ISO13321: Methods for Determination of Particle Size Distribution Part 8: Photon Correlation Spectroscopy, 1996.
[31] A. Rawle, Basic Principles of Particle Size Analysis, Technical Paper Malvern Instruments.
[32] C. Keck, R.H. Müller, Particle Size Analysis with Laser Diffractometry is not Sensitive Enough to Detect Changes in a Lipid Carrier System, AAPS, Nashville, 2005.
[33] C.M. Keck, R.H. Müller, Size analysis of submicron particles by laser diffractometry – 90% of the published measurements are false, Int. J. Pharm. 355 (1-2) (2008) 150–163.
[34] C.M. Keck, Cyclosporine nanosuspensions: optimised size characterisation & oral formulations, PhD thesis, 2006.
[35] R.H. Müller, S. Heinemann, Photon correlation spectroscopy and zeta potential characterization of model particles and colloidal drug carriers – essential information for the interpretation of cell culture studies, Biochem. Soc. Trans. 19 (2) (1991) 502.
[36] R.H. Müller, Zetapotential und Partikeladung in der Laborpraxis, Band 37, in: A. Paperback (ed.), Wissenschaftliche Verlagsgesellschaft mbH, Stuttgart, 1996.
[37] Malvern, Zeta Potential: An Introduction in 30 min, in: Application Note <http://www.malvern.co.uk>, MRK654 01.
[38] T.M. Riddick, Control of Colloid Stability through Zeta Potential, Zeta-Meter Inc. via Livingston Publishing Company, Wynnewood, 1968.
[39] L. Al Shaal, R.H. Müller, C.M. Keck, Production & stability of dermal hesperetin nanosuspensions, in: 5th German-Polish Symposium, New Challenges for Pharmaceutical Sciences, Poznan/Poland, 2009, P05.
[40] Schülke & Mayr GmbH, Euxyl K700 product description.
[41] T.F. Tadros, Applied Surfactants – Principles and Applications, Wiley VHC, Weinheim, 2005.