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Skin cell targeting with self-assembled ligand addressed nanoemulsion droplets

N. Atrux-Tallau*,†, T. Delmas†, S.-H. Han‡, J.-W. Kim§ and J. Bibette*

*Ecole Superieure de Physique et de Chimie Industrielles, ParisTech, Laboratoire Colloïdes et Matériaux Divisés, Université Pierre et Marie Curie, Paris 06, UMR 7195, 10 Rue Vauquelin, Paris 75231, France, †Capsum SAS, Heliopolis Batiment C, 3 allée des Maraichers, Marseille 13013, France, ‡Amore-Pacific Co. R&D Center, 314-1, Bora-dong, Giheung-gu, Yongin-si, Gyeonggi-do 449-729, South Korea and §Department of Applied Chemistry, Hanyang University, 55 Hanyangdaehak-ro, Sangnok-gu, Ansan, Gyeonggi-do 426-791,South Korea

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Synopsis

Dermo-pharmacy and cosmetic industries have utilized nanotechnologies for two decades. Initially proposed as vector systems for encapsulation of actives, they gained interest in increasing cutaneous bioavailability. Here, we assay the benefits of self-assembled nanoemulsions bearing ligands for targeting specific skin cells. Nanoemulsions, small droplets ranging typically from 20 nm to 150 nm, possess key properties for further use in cosmetics: long-term stability, optical transparency, extended range of textures and versatility. We investigated this nanoemulsion system and show ability to encapsulate a range of cosmetic actives with various physicochemical properties. Furthermore, this nanoemulsion presents a low cytotoxicity and is capable of directly targeting skin cells through simple addition of specific ligand in a one-step production protocol. This is of interest for increasing bioavailability of actives encapsulated into nanoemulsion droplets which may have penetrated the skin barrier to specific skin cell. Taken together, these chemical and *in vitro* observations suggest follow-up with *in vivo* models.

Résumé

L'utilisation des nanotechnologies en cosmétique et en dermo-pharmacie a commencée il y a vingt ans. Initialement simples vecteurs pour l'encapsulation d'actifs, de nombreux systèmes nanoparticulaires ont été développés afin d'améliorer la biodisponibilté cutanée des actifs. Dans notre étude nous proposons un système de nanoémulsion pour le ciblage spécifique de certaines cellules cutanées, dont les gouttelettes présentent des ligands auto-assemblés en surface. Les nanoémulsions, dont la taille des gouttelettes varie entre 20 et 150 nm, offrent de nombreux avantages dans leur utilisation en cosmétique : une stabilité prolongée, une transparence optique, une texture modifiable et une polyvalence d'emploi. Nous avons effectivement démontré la possibilité d'utiliser ces nanoémulsions afin d'encapsuler des actifs ayant des propriétés physicochimiques différentes, sans affecter la stabilité du système. De plus, ces nanoémulsions présentent une cytotoxicité minime et sont capables de cibler spécifiquement certaines cellules cutanées par simple ajout de ligands spécifiques lors de leur production en une étape. Ces nanoémulsions offrent un intérêt majeur dans l'amélioration de la biodisponibilité des actifs encapsulés en concentrant les gouttelettes ayant traversées la barrière cutanée sur les cellules cibles. Les caractéristiques physico-chimiques ainsi que les observations *in vitro* réalisées dans cette étude encouragent à tester ces nanoémulsions sur modèle *in vivo*.

Correspondence: Nicolas Atrux-Tallau, Laboratoire Colloïdes et Matériaux Divisés, ESPCI, 10 rue Vauquelin, 75231 Paris Cedex 5, France. Tel.: +33(0)1 40 79 58 40; fax: +33(0)1 40 79 52 45; e-mail: nicolas@capsum.eu.

Introduction

Emulsions, metastable colloidal dispersions made of two immiscible fluids, are mainly obtained through fragmentation of one phase into the other by applying suitable shear forces [1]. Droplets are stabilized through the use of surface-active agents, which prevent recombination during emulsification and ultimately ensure long-term metastability. Emulsions are indeed metastable systems which require energy for preparation in contrast to spontaneous emulsification of microemulsion compositions [2, 3]. Energy is thus necessary for creating excess surface, by finely dispersing one phase into the other [4]. Ageing of emulsion then occurs through two well-established mechanisms: (i) coalescence, fusion of droplets which requires nucleation of a hole within the thin continuous phase film surrounded by the two surfactant monolayers and (ii) Ostwald ripening, which leads to the enlargement of big droplets at detriment of small ones, through material exchange within the continuous phase [1]. Nanoemulsions are typically comprised of small emulsion droplets, from 20 to 150 nm, and can exhibit extended metastability (i.e. kinetic stability) as aqueous dispersion, as well as in more complex media. Indeed, their small droplets sizes prevent them from undergoing irreversible destabilization such as flocculation followed by coalescence. Because of these small sizes, pair interactions never exceed kT (kinetic energy), which precludes the occurrence of any liquid–solid-like phase transition (i.e. flocculation). Therefore, droplets contact duration upon Brownian collision remain sufficiently small to avoid coalescence nucleation. Thus, the main phenomenon supporting nanoemulsion ageing is Ostwald ripening. Because of the Laplace pressure exerted on droplets, the smaller ones tend to lose material in favour of bigger ones through diffusion into the dispersing phase [1, 4]. However, previous studies have demonstrated the existence of formulation rules allowing annealing the ripening effect [5–7]. Ostwald ripening can indeed be

efficiently prevented through entropic stabilization by trapped species encapsulation in both the core and at the membrane [4]. Specific heterogeneous interfacial film can, for instance, be used to stabilize nanoemulsion of low molecular weight oil, usually highly unstable. As a consequence, a fairly large oil composition domain can be explored. This is particularly interesting to finely tune products' sensory profile through mixing of low and high molecular weight oils. Low molecular weight oils provide a light and dry touch, whereas high molecular weight oils are usually experienced as heavy and greasy [8]. A wide range of textures can be obtained by varying the nanodroplets volume fraction, adding gelifying agents and/or mixing with other emulsion cream [9]. In addition, nanoemulsions are of particular interest for skincare products, being related to freshness, purity and simplicity by the consumer [8]. Finally, nanoemulsion adds large value to skincare products thanks to their good sensorial benefits such as an increased penetration and skin hydration [4].

Here, we capitalize on this background to explore the potential of these nanosystems in the field of cosmetics. Nanosizing and targeting are two well-explored routes to enhance bioavailability of drug, as very often reported for medical application, noticeably in chemotherapy by systemic route to overcome drug toxicity and adverse side effects.

Yet, nanoemulsion is also of particular interest for topical skin drug delivery: the passage of nanoemulsion droplets through the main skin barrier, stratum corneum and/or via the follicular ducts might i) selectively deposit in specific skin sites, ii) control and sustain the cutaneous drug release, iii) protect the drugs against substantial epidermal metabolism and iv) reduce the percutaneous absorption [10, 11]. Moreover, nanoemulsion is readily produced, scaled up, and the reduced sizes of the droplets confer translucent optical properties that are of interest for cosmetic applications [12].

Here, we use a previously developed and characterized nanoemulsion [9] to investigate its potential for cosmetic actives encapsulation, stability and targeted delivery to specific skin cells. This peculiar nanoemulsion is stabilized following the previously mentioned entropic stabilization [4]. Furthermore, the amorphous nature of the inner core is necessary to prevent cosmetic active expelling as well as ensuring long-term stability [9]. Finally, we explored the potential of such small droplets for targeting specific skin cells when active ligands are randomly added to the formulation during nanoemulsion production. Skin penetration of nanoemulsion droplets is limited by the barrier property of the stratum corneum, reducing availability of actives to skin cells. Targeting strategies should thus favour the limited number of nanoemulsion droplets that cross the skin barrier to concentrate on a specific cell type. Targeting strategies mainly employ specific ligands that are chemically grafted onto the surface of the carriers to increase bioavailability, decrease dose and thus, reduce side effects. However, ligand grafting requires heavy chemical reactions and creates molecules that require regulatory approval. To overcome this issue, recent studies focused on non-covalent strategies for addressing nanoparticles [13–15]. Similarly, taking advantages of our nanoemulsion system, we investigated the feasibility of targeting specific skin cells through direct ligand incorporation into nanoemulsions. More precisely, we selected a polypeptide lysine–threonine–threonine–lysine–serine (KTTKS) lipophilized with a palmitic acid for its physicochemical characteristics which should favour interface localization into the droplets. Peptide KTTKS is a subfragment of collagen I that demonstrated potent activity *in vitro* and *in vivo* on fibroblasts, favouring extracellular matrix synthesis and inhibiting collagenases activity (for review see [16]). Another targeting strategy was assayed with nanoemulsion droplets loaded with a trisaccharide pentacyclic triterpene, asiaticoside, usually extracted from Centella asiatica, a creeping perennial plant found in Malaysia and Asian countries [17]. Asiaticoside is active on skin cells and contains a trisaccharide chain glucose–glucose–rhamnose which may link to lectin type receptors [17]. Based on these reported observations, palmitoyl-KTTKS and asiaticoside are selected to target skin cells (i.e. keratinocytes, fibroblasts), through direct incorporation among nanoemulsion droplets preparation and compared to unloaded formulation.

Materials and methods

Nanoemulsion preparation

Nanoemulsion was prepared according to previously published work [9]. In brief, a lipophilic phase containing oil (soybean oil; Sigma-Aldrich, St Quentin Fallavier, France), wax (semi-synthetic glycerides, Suppocire NC, Gattefosse, France) and soybean phospholipids (Phospholipon 75, Lipoid, Germany) was prepared and homogenized through heating to 45°C. Meanwhile, the hydrophilic phase composed of polyethoxylated fatty acid (Myrj S40, Croda, France) in PBS 1X was made. Finally, both phases were crudely mixed, before properly emulsifying the system by high-energy process ultrasonication. The quantitative compositions were adapted to achieve lipophilic/hydrophilic surfactants ratios necessary to realize nanoemulsions with defined average size of 50 nm (50-nm NE) or 120 nm (120-nm NE) [4, 9]. The lipid fraction of nanoemulsion cores was composed of 50% w/w oil and 50% w/w wax mixture, known to prevent crystallization and to be in an amorphous state [9]. For long-term stability study, these formulations were diluted to a 10% w/w dispersed phase ratio, 0.2-μm filter-sterilized and split in aliquot for storage at room temperature, 45°C air oven and 4°C. Although the colloidal stability of these nanocarriers is a first requirement for their final application, these nanoparticles further need to be stable following their dispersion into a more complex system (e.g. aqueous gel, macroscopic emulsion or cream). Nanoemulsion stability in such formulae has thus been similarly evaluated.

Encapsulation of cosmetic actives

This nanoemulsion efficiently encapsulates a wide range of actives of different size and lipophilicity [18, 19]. For cosmetic application, we investigated the encapsulation behaviour of lipophilized peptide: undecenoyl dipeptide (DermaPep W220, Miwon, Korea), a stabilized vitamin (retinol-palmitate) and plant extracts (nimbin, seed extract from *Melia azadirachta*, camelina extract from *Camelina sativa* and hydrasalinol, lipophilic extract from *Salicornia herbacea*). Following nanoemulsion preparation with increasing concentrations of active, encapsulation was assessed by visual inspection, size measurement and stability against time. Maximum encapsulation was determined as the highest tested concentration of active for which nanoemulsion was formed and stable.

Self-assembled targeted nanoemulsion

Targeted nanoemulsion was obtained through direct incorporation of the ligand, palmitoyl-KTTKS from Creative Peptide (Shirley, NY, U.S.A.), into coarse emulsion, prepared as described previously,

followed by high-energy emulsification. The amphiphilic property of this ligand should expose the peptide head at the interface of the nanoparticles. Octanol/water partition coefficient of palmitoyl-KTTKS was predicted to be clog $P = 3.32$ [16]. Asiaticoside-targeted nanoemulsion droplets were prepared by direct incorporation of 1% w/w of total dispersed phase in coarse emulsion followed by high-energy emulsification. Asiaticoside octanol/water partition coefficient was estimated to be Xlog$P = 0.1$, favouring its interface localization. In both cases, nanoemulsion was achieved with a tip sonicator (Bioblock Scientific *VibraCell*. TM. *75042*) at 25% amplitude for 10 min with a 10 s on/30 s off cycle. Palmitoyl-KTTKS and asiaticoside concentrations correspond to a surface concentration of 0.03–0.06 and 0.1–0.15 peptide nm^{-2} for the 50-nm and 120-nm particles, respectively.

Physicochemical characterizations and stability

Nanoemulsions sizes were assessed by dynamic light-scattering measurement (NanoZS, Malvern, UK). Measurements were taken at a fixed angle of 173° using a 633-nm laser with each data point being the average of two independent measurements (15 runs of 10 s each). All samples were measured in 0.1 X PBS buffer at a dispersed phase weight fraction of 0.03 % to avoid multiple scattering effects. Average diameter and polydispersity were extracted from cumulant analysis of the autocorrelation function on an intensity basis. Zeta potentials were measured by electrophoretic light scattering (NanoZS). Data are the mean of two independent measurements (15 runs of 10 s each). For zeta determination, samples were diluted in 0.1 X PBS buffer at a dispersed phase weight fraction of 0.25%. Before measurement at 25°C, samples are left to equilibrate for 2 min.

Cell culture and reagents

Unless otherwise stated, cells and reagents were purchased from Life Technologies (Villebon sur Yvette, France). Human dermal fibroblasts (HDFa) were isolated from a 37-year-old woman, and keratinocytes from the HaCaT cell line [20] were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% v/v heat-inactivated foetal calf serum and 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin. Cells were incubated in a 37°C, 5% CO_2, humidity-saturated incubator. HaCaT passages were realized before cells reach confluency. Briefly, media were discarded from flasks, cells were washed with sterile PBS 1X, without calcium nor magnesium; then, 2 mL of trypsin/EDTA solution was added, and flasks were put back in the incubator for 3 min. Flasks were kept at room temperature until cells were round and detached from the flask surface. DMEM-FCS solution was then added to inhibit trypsin activity and remaining cells detached by trituration. Cells were centrifugated 7 min at 300 *g* and pellet was suspended in 1-mL DMEM-FCS for numeration and seeding. HDFa passages were realized at 80–90% confluence similarly to keratinocytes. However, trypsin activity was inhibited by adding to the cell suspension an equal volume of purified soybean trypsin inhibitor solution.

Cytotoxicity assay

Toxicity of nanoemulsions towards keratinocytes and fibroblasts was assessed by MTT assay. Briefly, 8500 cells per well were seeded in 96-well plates, cells were put back to the incubator for 48 h for passage recovery. Then, media were removed and cells exposed to increasing concentration of nanoemulsions in media without sera. Three hours before the 24-h contact period, 20 μg of MTT salt (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in 1X PBS was added to each well, and plates were incubated at 37°C and 5% CO_2 to complete the 24-h contact. Finally, media were carefully removed from wells, and formazan crystals were solubilized with 0.1% w/v Triton X100 in isopropanol. Absorbance was then read at 590 nm with a multiwell-plate spectrophotometer (SAFAS flx Xenius, Safas, Monaco). Cell viability results are presented as ratio of treated cells absorbance to untreated control cells absorbance.

Nanoemulsion cell adsorption assay

Ability of targeted nanoemulsions to adsorb onto skin cells was assessed *in vitro*. Cells were seeded in 8 chambers LabTek® slides (Fisher Scientific, Illkirsh, France) and placed in incubator for 48 h to allow recovery from passage. Then, media were replaced with 250 μg mL^{-1} nanoemulsion suspensions in media without FCS and incubated 1 h at 37°C, 5% CO_2. Following contact, cells were washed twice with 200 μL 1X PBS for 10 min, fixed with 200 μL 4% w/v paraformaldehyde in PBS 1X for 10 min and washed once with 200 μL 1X PBS. Finally, glass slides were separated from plastic chambers with the help of the provided apparatus, and slides were mounted with Fluoroshield – DAPI (Sigma-Aldrich). Observations were realized with a fluorescent microscope (Nikon Eclipse E600, Champigny sur Marne, France) equipped with Dil filters (G2A filters set, Ex 510–560 nm, DM 575 nm, BA 590 nm) (Nikon, Champigny sur Marne, France) and DAPI filters set (UV2A filters set, Ex 330–380 nm, DM 400 nm, BA 420 nm). Optical and fluorescent images were recorded with a CCD camera (Cascade 512B; Photometrics, Tucson, AZ, U.S.A.) driven by MetaVue software (Molecular Devices, Roper Scientific, Evry, France) in a similar acquisition configuration (e.g. gain 5 MHz, 100 ms exposure) to allow comparison and further quantification.

Results

Nanoemulsions formulation and stability

Nanoemulsions were successfully prepared according to Table I. First, the phospholipid surfactants are dispersed in the oil heated to 45°C. Meanwhile, the hydrophilic surfactants are dispersed in the phosphate-buffered water phase. The two phases are then crudely mixed and homogenized through ultrasonic cycles. Actives and ligands are added to these phases before emulsification depending on their respective hydrophilic or lipophilic nature. As expected, sizes of nanoemulsion droplets are close to 50 nm and 120 nm for unloaded formulae (Table I). Pal-KTTKS or asiaticoside loading did not significantly affect nanoemulsion droplets sizes even though they were slightly larger than unloaded (Table I). This suggests an interfacial localization of the ligands in-between phospholipids and PEG surfactants, modifying the interfacial film and thus particles' sizes. Zeta potentials were slightly negative and similar, with a mean zeta potential of -10.4 ± 2.2 mV whatever the size and the loading (Table I). Neither aggregation phenomena nor turbidity increase has been visually observed, and the size remains stable against time, even after a 1-year storage at room temperature for unloaded particles and pal-KTTKS or asiaticoside loaded (Fig. 1). Similar results were obtained at 4°C and 45°C (data not shown). Therefore, palmitoyl-KTTKS or asiaticoside incorporation at 1% w/w

Table I Mass compositions of nanoemulsion suspensions used for skin cell *in vitro* assays.

	50 nm NE	120 nm NE	50 nm Pal	120 nm Pal	50 nm Asia	120 nm Asia
Composition						
Wax	170	300	166.25	295.7	166.25	295.7
Oil	170	300	166.25	295.7	166.25	295.7
Phospholipids	65	45	65	45	65	45
Ligand	–	–	7.5	8.6	7.5	8.6
PEG-surfactant	345	215	345	215	345	215
Total mass	750	860	750	860	750	860
PBS 1X	1250	1140	1250	1140	1250	1140
Dispersed phase (%)	37.5	43.0	37.5	43.0	37.5	43.0
Physicochemical measurement						
Size (nm) (n)*	53.5 ± 6.6 (5)	116.7 ± 4.0 (5)	59.1 ± 11.7 (2)	128.9 ± 20.4 (3)	57.9 ± 10.2 (6)	132.1 ± 10.7 (6)
Polydispersity (a.u.)	0.123 ± 0.009	0.120 ± 0.004	0.128 ± 0.001	0.116 ± 0.005	0.177 ± 0.016	0.166 ± 0.037
Zeta (mV)	−8.9 ± 0.8	−10.2 ± 1.0	−8.7 ± 0.2	−9.8 ± 0.1	−10.1 ± 0.6	−14.8 ± 1.2

*Number of independent nanoemulsion preparations.

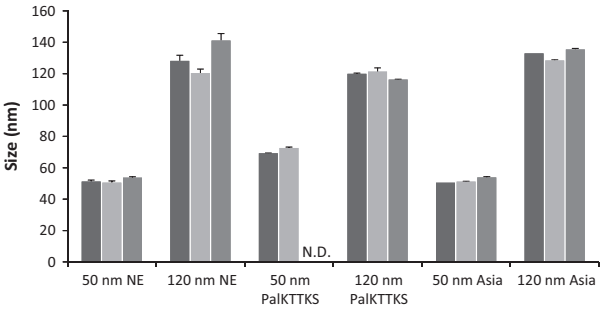

Figure 1 Size stability at room temperature of 50- and 120-nm nanoemulsions unloaded, loaded with palmitoyl-KTTKS or asiaticoside 1 % w/w of the dispersed phase. Data are the mean size of the particles ± SD determined by DLS measurements at day 0 (dark tone), following 4 months (light tone) and 1 year (medium tone).

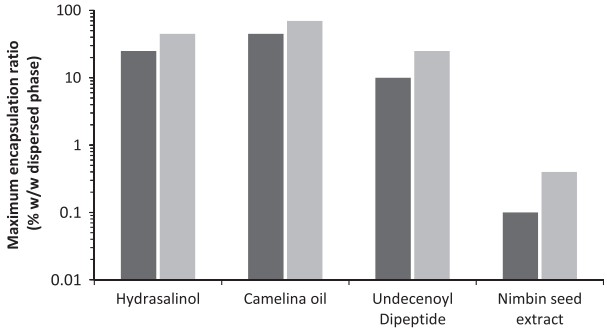

Figure 2 Maximum encapsulation ratio as a function of lipophilicity of encapsulated actives. Dark grey charts stand for the 50-nm nanoemulsions whereas the light grey charts stand for the 120-nm nanoemulsions.

of the dispersed phase does not destabilize the particles. The concentration achieves with these ligands corresponds to a density of 0.03–0.06 and 0.1–0.15 peptide nm^{-2} for the 50- and 120-nm particles, respectively.

Encapsulation of actives

Encapsulation efficiencies exhibit high differences depending on the nature of the actives. Although oil-like and wax-like materials such as Salicornia lipophilic extract and Camelina seed extract can be encapsulated up to 25% and 45% in the 50-nm NE particles and 45% and 70% in the 120-nm NE particles without instability, less lipophilic molecules, such as undecenoyl dipeptide, present an encapsulation limit of around 10% and 25% in the 50-nm and 120-nm NE formulations, respectively (Fig. 2). Finally, less lipophilic actives like Melia seed extract (log $P = 2.5$) present a low maximum encapsulation ratio of about 0.1% and 0.3%, respectively. However, asiaticoside, which is more amphiphilic (predicted log $P = 0.1$), was successfully loaded up to 2% without particle destabilization thanks to membrane localization (Data not shown). Lipid-like materials such as oils or waxes can, in fact, completely replace the nanoparticle core and be directly formulated with the required surfactants. As previously shown, the more lipophilic the active, the higher the maximum loading yield is. This has to be linked to the macroscopic solubility of the active in the lipid mixture, even though a partial relocalization of the active towards the membrane compartment could also occur [9, 16].

The effect of active encapsulation on nanoemulsion droplets stability was assessed by DLS size measurement as a function of time. Following 4 months at room temperature (summer and early autumn, Paris, France) or at 45°C air oven, the nanoemulsion systems containing actives remain stable (Data not shown).

Cell cytotoxicity of nanoemulsions

Human keratinocytes from the HaCaT cell line do not present dramatic viability outbreak after 24-h exposure to increasing concentrations of 50–120-nm-diameter nanoemulsion (Fig. 3a). More than 50% of the cells were viable after 24-h exposure to 500 $\mu g\ mL^{-1}$ concentrations as compared to untreated cells. 50-nm nanoemulsion tends to increase cell mortality as compared to 120-nm nanoemulsion; this may be due to higher surfactants content. Viability reduction is, in part, attributed to cell membrane

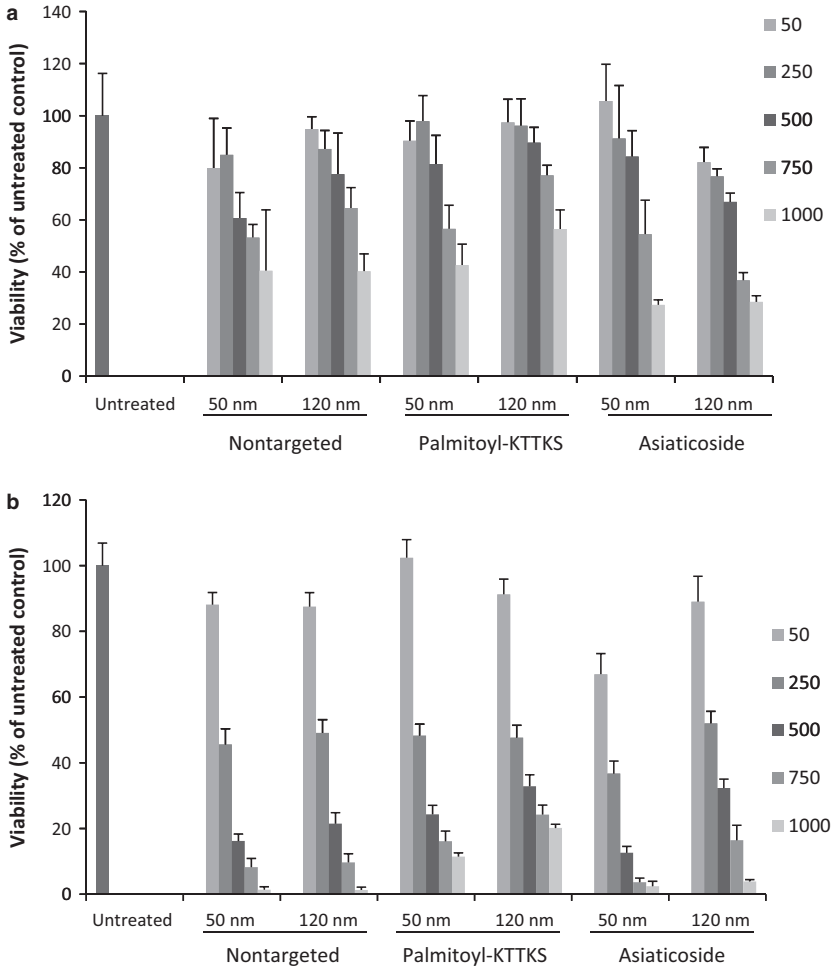

Figure 3 Nanoemulsions' cytotoxicity to keratinocytes of the HaCaT cell line (a) and normal human fibroblasts (b) Results are ratio of the mean +/− SD of six replicates to the untreated cells set to 100. Concentrations of 50, 250, 500, 750 and 1000 μg mL^{-1} were tested for 50-nm or 120-nm nanoemulsion non-targeted, loaded with palmitoyl-KTTKS or asiaticoside.

permeabilization as shown by propidium iodide assay (data not shown). Nanoemulsions containing palmitoyl-KTTKS lead to cell viability profile comparable to unloaded droplets. It nonetheless appears that the toxicity decreases with this targeting. This effect is statistically significant for 120-nm palmitoyl-KTTKS-loaded nanoemulsion versus non-targeted nanoemulsion at 750 and 1000 μg mL^{-1} concentrations. Keratinocytes treated with asiaticoside-loaded nanoemulsion present again a similar cytotoxic profile as compared to non-targeted nanoemulsion. Thus, cytotoxicity is mainly supported by nanoemulsion droplets than targeting ligands. Conversely, normal HDFa are more sensitive to the presence of nanoemulsion as half maximal inhibitory concentration (IC_{50}) is obtained with 250 μg mL^{-1}, whatever the formulation tested (Fig. 3b). Furthermore, observations of fibroblasts after treatment with nanoemulsion droplets show a decreased cell density which surely impedes with MTT cytotoxic assay results. Finally, for adhesion assay on cells and in accordance with IC_{50} results, nanoemulsions are used at 250 μg mL^{-1} for both keratinocyte and fibroblast cells.

Cell adsorption assay

Nanoemulsions were put into contact with keratinocytes and fibroblasts to determine whether direct incorporation of targeting compounds onto the droplets could promote their adhesion to cells. Upon dilution in media without sera, no aggregation phenomenon of nanoemulsion droplets was observed. Figure 4 synthesizes the observations realized on keratinocytes of the HaCaT cell line. Exposing keratinocytes to non-targeted nanoemulsion for 1 h leads to a perceptible fluorescence signal; this may be attributed to unspecific adsorption. Because HaCaT cells exhibit a colony growth pattern, it creates a network that could entrap nanoemulsion droplets, making washing steps less efficient. Thus, the fluorescence signal can be the conjunction of unspecific adsorption and spatial entrapment into the cell network. Normalized fluorescence intensities charts summarize all observations realized. Keratinocytes exposed to palmitoyl-KTTKS nanoemulsion do not show any discrepancies as compared to non-targeted nanoemulsion droplets. However, when keratinocytes are exposed to asiaticoside-targeted nanoemulsion,

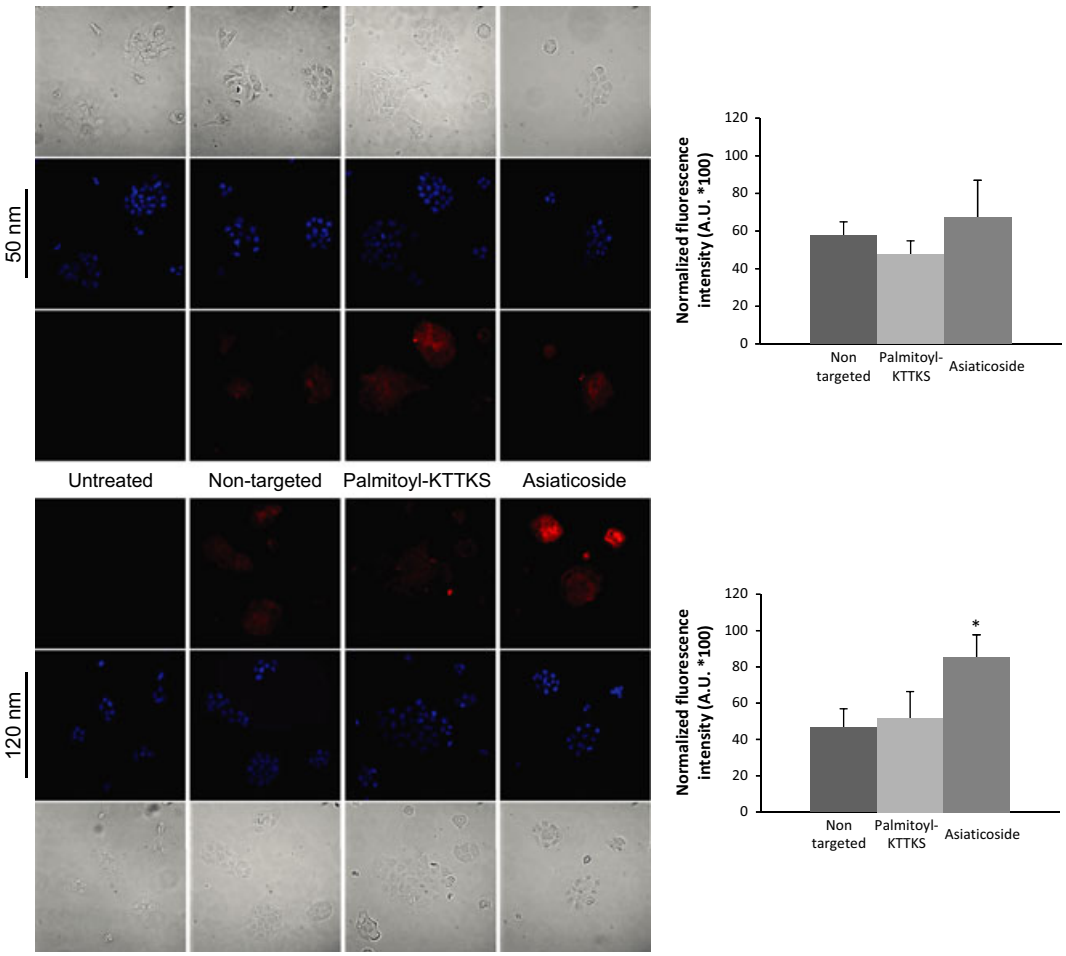

Figure 4 Selected acquisitions of nanoemulsions adhesion to keratinocytes from the HaCaT cell line and quantification of fluorescence intensities. Nanoemulsions of 50 nm and 120 nm were assayed, non-targeted or targeted with palmitoyl-KTTKS or asiaticoside. Acquisitions are split in bright-field view with corresponding DAPI-stained nucleus and fluorescence signal from nanoemulsion. Charts are the means ± SD of fluorescence intensities of nanoemulsion normalized by cell numbers in different acquisitions. (*$P < 0.05$ as compared to respective non-targeted control).

normalized fluorescence intensity increases and is statistically significant for 120-nm size nanoemulsion droplets as compared to non-targeted (ANOVA test, $F = 17.84$, d.f. = 1, 4, $P < 0.05$). Similarly, targeted nanoemulsions were tested on fibroblasts (Fig. 5). Normal HDFa grow as individual cells, making washing steps more efficient than with keratinocytes. Thus, unspecific adsorption is significantly reduced with non-targeted nanoemulsion even if a low autofluorescence signal may be visible. Even if no statistical significance was found because of variability of the results, from the different nanoemulsions tested, palmitoyl-KTTKS targeting appears to be the more efficient, giving a remarkable fluorescent signal on fibroblast cells.

Discussion

Our main focus concerns the use of nanoemulsions for active encapsulation and targeting. Previous studies have shown interest of this system to encapsulate highly lipophilic molecules for cancer therapy and diagnosis [18, 21]. We demonstrate that this nanoemulsion system similarly encapsulate cosmetic actives, with higher ratios for lipid-like material and highly lipophilic molecules such as natural oil and butter materials (e.g. camelina oil, hydrasalinol, undecenoyl dipeptide). Depending on nanoemulsion size, such actives can be encapsulated up to 45 or 70% w/w of the total dispersed phase for 50-nm and 120-nm particles, respectively. Loaded nanoemulsion droplets possess long-term stability comparable to the unloaded particles, for any lipophilic actives, as long as they are encapsulated below the maximum encapsulation ratio. Once formulated, the encapsulation stability mainly relies on the amorphicity of the lipid phase [22]. Indeed, other lipidic nanoparticles systems can initially encapsulate comparable amounts of molecular agents, but they often subsequently suffer from stability issues related to the crystallization of the lipid phase [23]. This usually leads to gelification of the system and expulsion of the encapsulated molecule [24]. Furthermore, varying core composition (i.e. wax/oil %w/w) while keeping an amorphous system allowed to control the internal viscosity, giving in turn the opportunity to control the release rate through diffusion [9, 25]. The more wax in the core,

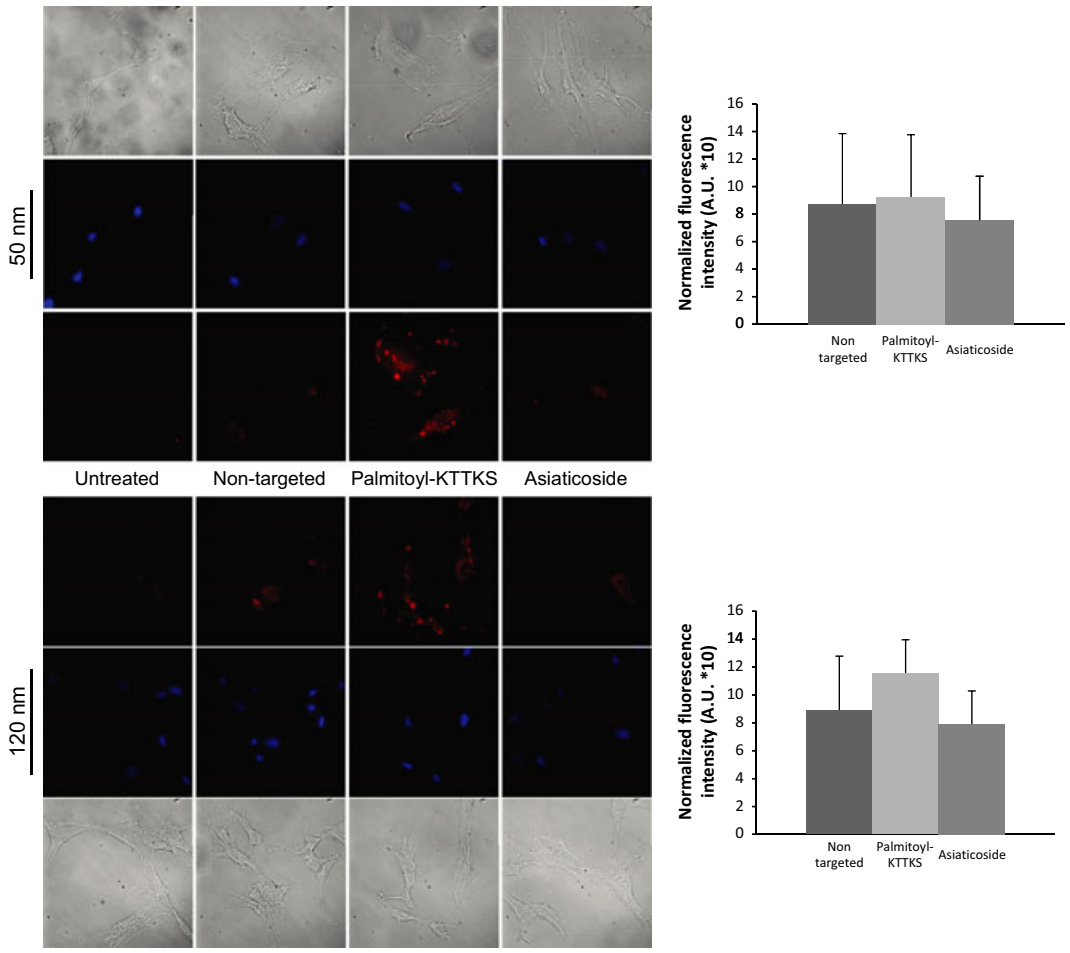

Figure 5 Selected acquisitions of nanoemulsions adhesion to normal human dermal fibroblasts (HDFa) and quantification of fluorescence intensities. Nanoemulsions of 50 nm and 120 nm were assayed, non-targeted or targeted with palmitoyl-KTTKS or asiaticoside. Acquisitions are split in bright-field view with corresponding DAPI-stained nucleus and fluorescence signal from nanoemulsion. Charts are the means ± SD of fluorescence intensities of nanoemulsion normalized by cell numbers in different acquisitions.

the more viscous the lipid matrix is and consequently the slower the release [25].

Originally developed for pharmaceutics, and more particularly for anti-cancer drug encapsulation and delivery, most systems rely on subsequent grafting of targeting molecules (i.e. peptides, biopolymers…) to the particle surface to increase bioavailability of the drug to the site of action [18]. Targeting usually requires grafting chemistry and therefore requires regulation approval for registration. Here, we propose an alternative to covalent binding by a non-covalent and self-assembled approach. Indeed, we show that direct incorporation of specific ligand, namely palmitoyl-KTTKS or asiaticoside, is sufficient for allowing nanoparticles targeting. A similar approach has been successfully developed utilizing the human serum albumin as nanocarrier non-covalently grafted with dye-labelled peptides [13]. We have shown that it is possible to produce targeted nanoemulsion with a one-step procedure relying on the direct emulsification of all components (the oil/wax mixture, surfactants and targeting ligand) by using high-energy processes. This approach allows simplifying the classical pharmaceutical approach by avoiding any chemical reaction. Consequently, no solvent is used and purification is not needed. Unloaded nanoemulsions and targeted nanoemulsion with palmitoyl-KTTKS or asiaticoside were tested on skin cells for cytotoxicity. Typically, lipid nanocarriers' toxicity mainly occurs through cytotoxicity of the surfactant used. Indeed, a good correlation exists between cytotoxicity of lipid nanocapsules and cytotoxicity of pure surfactant used [26]. A recent study showed that microemulsions and solid lipid nanoparticles stabilized with phospholipids did not present dramatic cytotoxic effect on fibroblasts 3T3 cell line nor keratinocyte HaCaT cell line [27]. We find that human fibroblasts are more sensitive to nanoemulsion treatments with an IC_{50}, which is half IC_{50} obtained for HaCaT cells. As previously observed, culture of primary human cells is generally more sensitive than their corresponding cell line, as evidenced for normal human fribroblasts compared to the 3T3 fibroblasts cell line [28].

Targeting abilities of our loaded nanoemulsions were assessed through adhesion assay. Cells were treated during 1 h with 250 μg mL^{-1} of nanoemulsions stained with a fluorescent probe.

KTTKS peptide is a fragment of pro-collagen that is known to stimulate extracellular matrix synthesis through undefined mechanism of action, which may imply a specific interaction with fibroblasts [16, 29]. The palmitoyl derivatization of this pentapeptide enhances skin delivery and bioavailability with similar activity than the native pentapeptide [16]. The amphiphilic nature of palmitoyl-KTTKS suggests a possible interfacial localization during nanoemulsion droplets assembly, thus favouring interaction with cells. It appears that palmitoyl-KTTKS favours nanoemulsion droplets adhesion to dermal fibroblasts cells. This adhesion is clearly visible as compared to non-targeted nanoemulsion droplets or asiaticoside-targeted nanoemulsion. This is of particular interest considering that palmitoyl-KTTKS was directly incorporated into the nanoemulsions droplets. As a first hypothetical explanation, we measured the zeta potential, which is similar upon the different formulae; however, no change was detectable, which may suggest that the adhesive mechanism is more complex that simply related to electrostatic forces. Concerning asiaticoside-targeted nanoemulsion droplets, we demonstrated a preferential binding on keratinocyte cells. This binding was even significant with 120-nm droplets as compared to non-targeted nanoemulsion droplets. Again, the zeta potential of the different formulae is similar, which is not suggesting a simple electrostatic interaction with the cells. Most often, Centella asiatica extracts and asiaticoside are assayed on fibroblasts and have been reported to stimulate type I collagen synthesis in fibroblasts culture as well as fibronectin synthesis [30]. Asiaticoside also suppress keloid-derived fibroblasts proliferation and collagen synthesis [31]. Furthermore, C. asiatica extracts inhibit keratinocytes proliferation, which is of interest in hyperproliferative skin disorders such as psoriasis [32]. We here demonstrated that asiaticoside most likely interacts with keratinocyte which does not exclude a possible action on fibroblasts through internalization of asiaticoside.

Conclusion

We successfully encapsulated in nanoemulsion droplets a broad range of cosmetic actives with different physicochemical characteristics. Upon encapsulation of actives, we were able to control nanoemulsion droplets sizes in a narrow range. Targeted nanoemulsion droplets and active loaded nanoemulsion were stable against time up to 1 year. Interestingly, we demonstrated that increased adhesion of nanoemulsion droplets onto skin cells can be achieved through direct incorporation of specific ligand during emulsification. Upon the selected ligand (e.g. palmitoyl-KTTKS or asiaticoside), different skin cell type may be targeted. This is of interest for increasing bioavailability of actives encapsulated into nanoemulsion droplets which may have penetrated the skin barrier. Taken together, these chemical and *in vitro* observations suggest follow-up with *in vivo* models.

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