Natural lipids in nanostructured lipid carriers and its cytotoxicity

Paula A. Lima[1], Caroline A.D. Rampazo[1], Amanda F. Costa[1], Tiago Rodrigues[2], Carolina M. Watashi[2], Nelson Durán[1,3,4,*]

[1]Institute of Chemistry, Biological Chemistry Laboratory, University of Campinas (UNICAMP) - Campinas - SP, Brazil
[2]Center of Natural and Human Sciences, Federal University of ABC, Santo André, SP, Brazil, [3]NanoBioss (UNICAMP), SP. Brazil
[4]National Nanotechnology Laboratory (LNNano-CNPEM), Campinas, SP, Brazil

*duran@iqm.unicamp.br

Abstract. Nanostructured lipid carriers (NLCs) are active carrier systems which modulate the sustained release of actives and protect unstable compounds against degradation. NLCs can also protect skin from sun light, due to its particulates nature, which gives them intrinsic scattering properties. In this work, we present the preparation of NLCs using natural lipids and its cytotoxicity profile. It was used a vegetal butter with melting point (m.p.) ~32-40°C, an animal wax (m.p. 35-40°C) and a vegetal oil (boiling point ~120-150°C). NLCs were prepared by hot high pressure homogenization method and particles were characterized by average size (Z_{ave}), polydispersity index (PDI) and zeta potential (PZ) (Fig.1). The thermal behavior of the NLCs was studied using Differential Scanning Calorimetry (DSC). All the formulations were followed up for 60 days in order to evaluate their stability. NLCs exhibited a Z_{ave} around 150-200 nm, PDI less than 0.2 and PZ varying from -25 to -40 mV. The m.p. for the lyophilized NLCs was about 40-56°C. Cytotoxicity of the formulations were evaluated for human keratinocytes (HaCaT) and melanocytes (Melan-A) in the exponential growth phase. Cell viability was used as indicator of cytotoxicity and determined after 4 days of culture by MTT assay. It was found that the NLC formulations were not toxic against HaCaT and Melan-A cells. Results showed that the NLCs produced are potential carriers for nanocosmetics and sunscreen products.

1. Introduction

Although ultraviolet (UV) radiation is only around 5% of the total radiation emitted by the sun, UV radiation is mainly responsible for causing damage to human health [1], increasing the risk of various skin diseases such as sunburn, photo-aging and cancer [2]. The biological effects of UV radiation vary widely with wavelength and therefore, the UV spectrum is divided into three regions: UVA (320-400 nm), UVB (290-320 nm) and UVC events (100 - 290 nm) [1]. While UVC radiation is absorbed by the ozone layer in the stratosphere, the UVA and UVB radiation can reach the Earth's surface, affecting humans and ecosystems [3].

At sea level, the UVA radiation comprises about 95% of the UV radiation emitted by the sun and is mainly responsible for the persistent darkening of the skin, besides being related to premature aging and disease [3-5].

The use of TiO_2 as an active sunscreen ingredient (loading around 2-15%) for a long time has raised concerns about potential risks from the generation of free radical formation. To date, remediation attempts have concentrated on reducing the yield of free radical generation by TiO_2 upon sunlight exposure. Many new strategies are continuously developed, such as nontoxic, biocompatible shell that

neutralizes the free radicals by scavenging them with natural antioxidants before they exit the particle. This is interesting but is costly to the cosmetic industry [5´].
When Ti_2O compared with other common solar protector, as benzophenone-3 (BZ-3), the latter is biodegradable, Ti2O is not.

Although benzophenone-3 or oxybenzone (BZ-3) is used as an active ingredient in sunscreens for over 40 years [3], it has a high incidence of photoallergies' effects [4,5]. Some studies relate to BZ-3 absorption of endocrine problems and experiments using *in vitro* and on animal showed that BZ-3 influences the reproduction and the action of sex hormones. Furthermore, it is suspected that the BZ-3 influence the appearance of hormone-dependent diseases [3].
The toxicity of BZ-3 is related to the degree of penetration into the skin, which depend strongly on the nature of the vehicle in which the formulation is prepared. Properties such as solvent polarity, particle size and type of vehicle used are important in sunscreen behavior on the skin surface [2]. Thus, the development of formulations that prevent the penetration of the skin BZ-3 is a challenge for the cosmetics manufacture.
A strategy to prevent the penetration of active ingredients on the skin can be encapsulation in nanometric particles. Among them, the so-called solid lipid nanoparticles (SLNs) were developed in the early 1990s as an alternative to the system such as, emulsions, liposomes and polymeric nanoparticles. The SLNs are produced by substituting the liquid lipid (oil) of an oil / water emulsion of a solid lipid or a mixture lipids, so that the lipid matrix is solid at room temperature and body. Lipids are stabilized by 0.5 to 5% (w / w) of surfactant [6]. While these have good stability and low toxicity [7], the main disadvantage of SLNs is its low loading capacity, ranging from 25-50% depending on the solubility of the active lipid matrix method used in the preparation and polymorphic state of the lipid matrix [8].

The second generation of such material is called nanostructured lipid carriers (NLCs) and they improved the loading capacity. Lipid nanoparticles made from nontoxic lipids are potentially good systems for the carrying of sunscreens. Furthermore, the lipid nanoparticles act as physical sunscreens, due to their nanoparticulated characteristic. This effect is important because it is possible to reduce the amount of active encapsulated without compromising the effectiveness of the sunscreen, but reducing the risk of side effects [9].

Vegetable lipids are ingredients commonly found in cosmetics because of its moisturizing properties and therefore, are strong candidates in the production of NLCs. One is the cupuaçu (*Theobroma grandiflorum*) from Amazon. The seeds contain several fats and can be utilized in the extraction of butter, which is used in food and cosmetics [10]. The cupuaçu butter has a balanced composition of saturated and unsaturated fatty acids, which gives it a low melting point, facilitating the contact with skin. Moreover, it has high power of water absorption, an important feature in the preparation of cosmetics [11]. Lanolin (m.p. 36.1^{o}C) is also another solid fat that are usually used in NLC [12].

The Buriti oil from *Mauritia flexuosa* palm native of South America exhibits rich composition in unsaturated fatty acids such as omega-6 (linoleic acid), known by the potential to reduce the effects of oxidative stress. Furthermore, it shows high β-carotene content and α-tocopherol (antioxidant) and a large oxidative stability [13].
This work aims to produce and characterize nanostructured lipid carriers for the encapsulation of benzophenone-3, using natural lipids cupuaçu butter, lanolin and oil buriti, aimed at use mainly as sunscreens, exhibiting less toxicity than the BZ3 alone.

2. Methods

2.1. Reagents

The materials were obtained from different sources (previosly being notified to PatGen-Brazil): Cupuaçu butter (courtesy from Inovam Brazil); - Buriti Oil (Beraca); - Lanolin Anhydrous U.S.P.

(Courtesy from Croda Brazil); - Pluronic F68 (Aldrich); - Benzophenone-3 (Aldrich); - Methanol HPLC grade (Merck); - Potassium chloride (USB); - Carbopol 940 (Synth).

2.2. Preparation of nanostructured lipid carriers (NLCs) by high pressure homogenization (HPH)

The preparation of NLC was performed by high pressure homogenization (HPH). It was used a methodology adapted from Durán et al. [14] and Durán and Deni [12]. Briefly : A mixture of 3-4 g of cupuaçu butter, 1-2 g of lanolin and 1-3 g of Buriti oil was melted at about 45^{o}C. In the samples was added benzophenone-3 and waited until complete solubilization. The mixture was then poured into 300 ml of an aqueous solution of 0.3-0,7% of the stabilizer Pluronic F68 at the same temperature, under stirring of 10,000 rpm in an Ultra-Turrax T18. The mixture was kept under stirring for about 2 min. The obtained pre-emulsion was immediately added to the high-pressure homogenizer (Panda 2K, Niro Soavi). Then, 2-5 homogenization cycles were performed, with pressures of 400-800 bar. Next, the obtained formulation was cooled in an ice bath to room temperature to give the NLCs. Part of the dispersion was stored in a refrigerator (temperature around 4°C) and the other at room temperature for comparison of the effects of storage temperature stability.

2.3. NLCs characterization and stability study

2.3.1. Size of particles, polydispersity index and zeta potential

The average particle size (Z_{ave}) and polydispersity index (PDI) of NLCs were analyzed by dynamic light scattering technique (DLS) using a Nano ZS Zetasizer device Malvern, at 25^{o}C. The dispersions CLNs were diluted in deionized water at 1: 100 and analyzed in polystyrene cuvettes. The zeta potential of the dispersions was obtained using equipment Nano ZS Malvern Zetasizer, at 25^{o}C. Samples were diluted at 1: 100 with 1 mM KCl. All analyzes were performed in triplicate and the formulations were followed for up to 75 days.

2.3.2. Dispersion pH monitoring

The pH of CLNs dispersions were measured using the pH meter Qualxtron QX 1500 Plus, which was calibrated using commercial buffer pH 4 and 7. The formulations were followed for up to 75 days.

2.3.3. Measurement of encapsulation efficiency (EE%) and loading capacity (LC%)

The encapsulation efficiency and loading capacity of NLCs were estimated by spectrophotometer in the UV and visible regions, employing a AJ Micronal AJX-6100PC equipment. Initially, it was constructed a calibration curve of benzophenone-3 in calibration solution of methanol / water 90:10, and analyzed at wavelength 324 nm. The analyses of the patterns were carried out in triplicate.
For the preparation of the samples, it was used an adapted methodology from Luan et al. [15]. For % EE and LC% calculations, two types of sample were prepared in order to calculate the total mass of the suspensions of BZ-3 (M_T) and the mass encapsulated in nanoparticles (M_E). The total mass of the suspensions BZ-3 was estimated as follows: a known volume of the formulation was transferred to a 25 ml volumetric flask, where the volume was measured so that the ratio methanol / water was equal to 90:10 . The resulting suspension was brought to a Branson 1510 ultrasonic bath for 30 min to cause breakage of the particles. Then the samples were centrifuged in a Eppendorf 5804R centrifuge for 30 min at 5000 rpm to cause the sedimentation of the lipid fraction. The obtained solutions were analyzed by UV-vis spectrophotometer at a λ 324 nm. The analyses were performed in duplicate.
The BZ 3-encapsulated mass was determined as follows: A known volume of formulation was filtered using a microfilter centrifuge with 10 kDa cut mass in an Eppendorf centrifuge 5424-14000 rpm for 40 min. Next, the filtered lipid fraction was removed from the microfilter inverting it in a vacuum

microfilter and subjecting to centrifugation at 14000 rpm for 40 min. The solid was transferred to a 25 ml volumetric flask, which was measured using a solution of methanol / water 90:10. The resulting suspension was brought to a Branson 1510 ultrasonic bath for 30 min to cause solubilization of BZ-3 in methanol solution. Then the samples were centrifuged in a Eppendorf 5804R centrifuge for 30 min at 5000 rpm to cause the sedimentation of the lipid fraction. The obtained solutions were analyzed by UV-Vis spectrophotometer at λ 324 nm. The analyses were performed in duplicate.
The encapsulation efficiency calculations (EE%) and loading capacity (LC%) equations were performed by using equations 1 and 2. Where T_M is total mass of BZ-3 in suspensions, M_E is the mass encapsulated in nanoparticles and $M_{lipidic}$ is the mass of lipids used.

$$\boldsymbol{EE}\% = \frac{\boldsymbol{M_E}}{\boldsymbol{M_T}}\mathbf{100}\% \qquad (1)$$

$$\boldsymbol{LC}\% = \frac{\boldsymbol{M_E}}{\boldsymbol{M_{lipídica}}} \qquad (2)$$

2.3.4. Lyophilization-redispersion assays

Assays were performed by freeze (lyophilization)-re-dispersion with NLC samples without BZ-3 (CLN0) in the absence and presence of trehalose (2 to 5%), and NLC samples containing 10% BZ-3 (CLN10) in the absence and presence of glucose 5-30%. Samples were lyophilized and re-dispersed subsequently using ultrasound bath for 30 min. To assess the conservation of the nanoparticles after a freeze-re-dispersion cycle, average particle size measures and zeta potential were performed.

2.3.5. Differential Scanning Calorimetry (DSC) measurements

DSC analyzes were conducted using previously lyophilized samples. It was used a TA Instruments Q10 DSC equipment, operating in the temperature range 5°C to 55°C at a heating rate of 5°C min^{-1} under Nitrogen.

2.4. Cytotoxicity

The tetrazolium reduction assay was done as described by Denizot and Lang [16]. Briefly, 0.1 ml of serum–free medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) was added to each well. After incubation of NLCs for 4 h, the supernatant was removed and the blue formazan product obtained was dissolved in 0.1 ml of ethanol with stirring for 15 min on a microplate shaker after which the absorbance was read at 570 nm, using for human keratinocytes (HaCaT)(CLS Heidelberg, Germany) and melanocytes (Melan-A) (obtained from Dr. Dorothy Bennett, St. George's Hospital, UK) in the exponential growth phase.

3. Results and discussions

The high pressure homogenization (HPH) is a technique used for years for the production of nanoemulsions for parenteral nutrition. For the production of LSNs and NLC, HPH is a reliable and powerful technique, and easily scalable for large quantities [17]. In addition, HPL leads to homogeneous products, with low tendency to aggregation and therefore more stable [18].

NLCs produced showed on the day of production, average diameter of 173.8 ± 1.9 nm with no noticeable agglomerates of particles in the micrometer size range. The NLC0 (without active) presented PDI less than 0.2 in all measures, indicating that the system has low polydispersity. Due to the low polydispersity of the produced system, the aging Ostwald can be avoided in the long term [19]. On the day that it was produced, the NLC0 presented PZ = -38.3 ± 0.9 mV, and a high value may indicate good stability related to the aggregation processes.

The physicochemical stability of the formulation in the long term is an important parameter to assess the feasibility of application of carrier systems. The NLC0 was analyzed over 154 days. It was observed subtle changes in Z_{ave} values, indicating that no significant aggregation of the nanoparticles in this period. However, for the zeta potential, there was a gradual decrease in the zeta potential module, reaching a peak of -26.6 ± 5.4 mV after 64 days of storage for samples kept at 4°C, and -31, 8 ± 5.6 mV for the specimen kept at room temperature. Next, there was an increased zeta potential module followed by a stabilization at values close to -35 mV. Zeta potential changes may be related to changes in the crystalline materials network without, however, causing the particle average size changes.

Monitoring of the pH of the NLC0 formulation showed a small variation in pH during the period of 154 days (4.77 to 4.95). A low variation in the pH is related to the chemical stability of the system, so that the NLC0 shows good chemical stability during the analysis period that is an important feature for the application of cosmetic formulations.

Moreover, it is observed that the dispersions stored at 4°C and at room temperature showed the same trends during the storage period at all parameters. Thus, the NLCs produced were stable in the long term (about 5 months) even when stored at room temperature.

The NLC2,5-15,0 (5-15% percentage of BZ-3) had, after their preparation, Z_{ave} = 175.8 ± 2.0, 178.5 ± 3.8, 166.7 ± 0.62 and 163.5 ± 3.0 nm, respectively (Table 1). It was observed that there were no significant differences in average particle size when NLC2,5 and NLC5,0 containing BZ-3 were compared to NLC0. The samples showed NLC10,0 and NLC15,0 Z_{ave} about 10 nm and smaller than NLC2,5 and NLC5,0, possibly due to the greater amount of encapsulated BZ-3, which should lead to changes in the crystalline structure of the nanoparticles. The polydispersity indexes obtained for the NLCs were lower than 0.2 on the preparation of these formulations, which indicates to obtain monodisperse samples with low tendency to Ostwald aging [19].

It was observed that, during the storage periods, there were few changes in average size of particle, which indicates that there was no formation of aggregates. This feature is important for the development of cosmetics, since the storage at room temperature brings less handling difficulties with products.

The zeta potential obtained for the NLC2.5 to 15.0 on the day of preparation were -21.1 ± 1.5, -20.0 ± 2.7, 26.8 ± 2.6 and -34, 3 ± 1.3 mV, respectively (Table 1). All values obtained for the NLCs containing BZ-3 were less negative than those obtained for the CLN0, due to possible changes in the external surface of the nanoparticles. However, steric effects are also responsible for the stability of NLCs. For systems in which there is a combination of steric and electrostatic effects, as in the case of NLCs produced in this work a zeta potential of ± 20 mV may indicate be stable [20].

The monitoring of NLCs during the storage time shows that small changes occur in Zeta potential of the formulations. In general, the NLCs stored at 4°C and at room temperature showed similar trends of zeta potential and pH in the studied periods. For all formulations containing BZ-3, Zeta potential remained in the range between -17 and -43 mV. The observed changes were due possibly to structural changes, such as the expulsion of BZ-3 to the outer regions of nanoparticles or penetration into the innermost regions. The BZ-3 interaction with the surfactant Pluronic F68, the surface of the nanoparticles could also be a factor causing changes in the surface charge and thus the zeta potential.

During the storage period, the zeta potential of most NLCs presented to more negative than -20 mV, indicating that the formulations exhibit good stability.

On the first day, NLC2,5-15,0 had pH 5.03-5.36, respectively. Then, no significant changes were observed in the NLCs pH during storage period, which can indirectly indicate that the formulations are chemically stable.

The encapsulation efficiency and entrainment capacity are important characteristics to assess the implementation feasibility of a carrier system for certain targets. In this study, both were determined using an indirect method consisting of the comparison between the amount of BZ-3

encapsulated in lipid nanoparticles, and the total amount of the colloidal system, in accordance with equations 1 and 2.

The BZ-3 is an active desirable as sunscreen exhibiting a protection factor (SPF) greater than 15, due to their strong absorption in the regions of UVA (320-400 nm) and UVB (290 - 320 nm) [21].

Quantification of BZ-3 in NLCs the samples were performed through rupture of nanoparticles by sonication using methanol, which is capable of solubilizing BZ-3 without any significant solubilization of the lipids present in the sample.

Table 1 shows the results of EE% and LC% obtained for all the NLCs2.5-15 after production. High encapsulation efficiencies were obtained for all NLCs produced, varying between 87.2 and 91.0%. The high efficiency of encapsulation can be attributed to the high concentration of liquid lipids and the complex mixture of solid lipids, which generate large imperfections in the crystal lattice of lipids, which serve as spaces for encapsulating the asset. Furthermore, BZ-3 should have high solubility in liquid lipids [22].

Table 1. Encapsulation efficiency (EE%) and loading capacity (LC%) of NLCs 2.5-15.0 and their physicochemical properties.

Formulation	% of BZ-3	EE%	LC%	Z_{av} (nm)	Zeta Potential (mV)
NLC2.5	2.5	90.8	2.4	175.8 ± 2.0	-21.1 ± 1.5
NLC5.0	5.0	91.0	4.8	178.5 ± 3.8	-20.0 ± 2.7
NLC10.0	10.0	87.2	9.1	166.7 ± 0.6	-26.8 ± 2.6
NLC15.0	15.0	87.5	14.4	163.5 ± 3.0	-34.3 ± 1.3

For the viability of developed formulations, it is important that the active is not released during storage, i.e. the %EE should not change by this time. The encapsulation efficiency and loading capability of NLC2.5 and NLC5.0 formulations were reanalyzed after 48 days storage at 4°C or at room temperature.

According to the data, there was a small increase in EE% of NLC2.5 and NLC5.0 formulations during storage. Furthermore, there was no significant difference between the NLCs maintained for 48 days at 4°C and room temperature, which indicates that storage of the formulation can be done at room temperature. This feature is very important in the development of products with possible applications cosmetic due to the good conservation of the cosmetic characteristics of the ambient conditions.

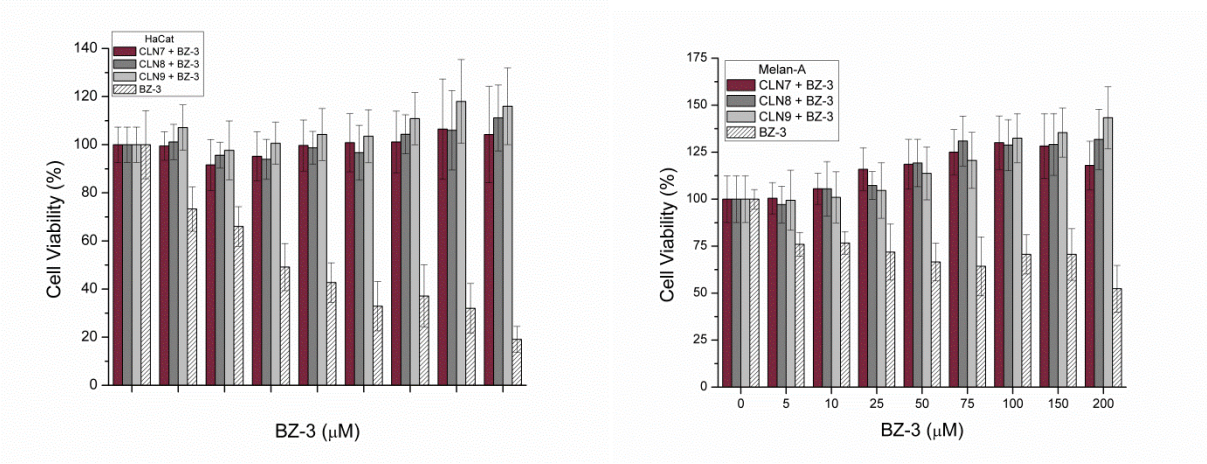
The results are consistent with other systems containing 3-BZ. Lacerda et al. [23] obtained NLCs from carnauba wax and isodecyl oleate by high pressure homogenization with 91% encapsulation efficiency for formulations containing 48 μg BZ-3 per mg of lipids. However, after 30 days of storage EE% of these systems decreased to 58.8%. The complexity of the mixture of lipids contained in NLC2.5 and NLC5.0 produced in this work, it is probably responsible for the increased stability of the encapsulation, due to increased disorganization of the crystalline lattice.

Lyophilization-re-dispersion tests were performed in order to evaluate the behavior of nanoparticulated systems after dehydration by lyophilization. For this analysis was performed using NLC0 and NCL15.0 and in the presence or absence of cryoprotectant, which would function preventing agglomeration of the nanostructured carriers. For all samples, after the addition of water and sonication, it was observed the formation of a gel lumpy appearance, unlike the prior lyophilization homogeneous dispersion. Particle size measurement and polydispersity index performed under the same conditions used in the NLCs analysis does not freeze, showed results which indicated the presence of large agglomerates and high polydispersity, which indicates that lyophilization-re-dispersion does not produce equivalent systems these early. Therefore, the storage of NLCNs tested in lyophilized form was not suitable due to not ensure the maintenance of the characteristics of systems.

The results obtained in DSC analyses show that the melting temperature ranges for NLCs from 2 to 40% liquid lipid varied very little from each other. Furthermore, the fusion of NLCs occurred within a wide temperature range, which was consistent with the fact that the NLCs were composed of a complex mixture of lipids, and not to a pure substance.

Subtle differences were observed between the melting points of the different NLCs, which indicates the possibility of using formulations with up to 40% liquid lipid. It is assumed that, the larger the amount of the lipid, the greater the benefits the NLC presented at the end, since this oil has a high composition linoleic and palmitic acids, giving it the character of a non-drying oil, e.g., prevents dryness [24]. However, as a greater amount of oil in the formulation generates particles with a larger average diameter.

Cytotoxicity of the formulations was evaluated for human keratinocytes (HaCaT) and melanocytes (Melan-A) in the exponential growth phase. Cell viability was used the indicator of cytotoxicity and determined after 4 days of culture by MTT assay (Fig.1 ab). It was found que the NLC formulations were not toxic against HaCaT and Melan-A cells. However, the free BZ-3 was toxic to HaCaT cells (IC_{50} of ~ 25 μM) and to Melan-A was less toxic (IC_{50} of ~ 200 μM).



(a) (b)

Figure 1. Cell viability of HaCat cells (a) and Melan-A cells (b) treated with encapsulated BZ-3 and its free form.

4. Final remarks

Nanostructured lipid carrier's formulations were produced containing BZ-3 through the high pressure homogenization technique. The NLCs produced had average particle sizes around 175 nm and low polydispersity index, which indicates that the samples were approximately monodisperse. The zeta potential of the produced NLC was compatible with systems steric and electrostatic stabilized. The monitoring of NCLs produced at 4°C or at room temperature showed that the formulation remained stable in storage. It was observed the presence of aggregates and relatively small changes were observed in the pH and zeta potential of nanoparticles, which should be related to structural changes in particles that may have occurred during the storage period.

It was observed that there were no significant differences between samples stored at 4°C and room temperature, so that the storage of NLCs at room temperature is possible. This feature is desirable in the development of cosmetics. Finally, this nanostructured sunscreen appears biodegradable and more stable than the common sunscreen, as TiO_2, that is free radical producer and non biodegradable.

Acknowledgements

Support from FAPESP, CNPq, INOMAT (MCTI/CNPq), NanoBioss (MCTI) and Brazilian Network on Nanotoxicology (MCTI/CNPq) are acknowledged.

References

[1] Diffey BL 2002 *Methods* **28** 4
[2] Gulbake A, Jain A, Khare P and Jain SK 2010 *J. Microencapsul.* **27** 226
[3] Kim S and Choi K. *Environ. Inter.* 70, 143–157 (2014).
[4] Sambandan DR and Ratner D 2011 *J. Amer. Acad. Dermatol.* **64** 748
[5] Lautenschlager S, Wulf HC and Pittelkow MR 2007 *Lancet* **370,** 528
[5´] Morsella M, D'Alessandro N, Lanterna AE, Scaioano JC 2016. *ACS Omega* **1,** 464
[6] Pardeike J, Hommoss A and Aiman RH 2009 J. *Inter. J. Pharm.* **366** 170
[7] Poulsen HS, Kristensen NHG, Heydenreich AV and Pedersen N 2006 *Eur. J. Pharm. Biopharm.* **62** 155
[8] Weber S, Zimmer A and Pardeike J 2014 *Eur. J. Pharm. Biopharm.*. **86** 7
[9] Puglia C, Bonina F, Rizza L, Blasi P, Shoubben A, Perrotta R, Tarico MS and Damiani E 2012 *J. Pharm. Sci.* **101** 301
[10] Genovese MI, Trunchado P, Tomar-Barberan FA and Pugliese AG 2013 *J. Agric. Food Chem.* **61** 2720
[11] Mancini-Filho J, Torres RP, Solis VS, Alvarado LF and Vasquez-Ocmin PG 2010 *Grasas y Aceites* **61** 390
[12] Durán N and Deni, AXP 2015 *Brazilian Patent PIBr*. 102014028002 2; PCT/BR2015/000121
[13] Moura A, Oliveira MM, Rangel JHG and Aroucha I 2010 Congresso de pesquisa e inovação da rede norte nordeste de educação tecnológica, N. **5**, Maceió. Artigos. Maceió: CONNEPI.
[14] Durán N, Caverzan J and Marcato PD 2013 *Brazilian Patente. PIBr*. 102013033412-0
[15] Luan J, Zheng F, Yang X, Yu A and Zhai G 2015 *Colloids Surf. A: Physicochem. Eng. Aspects* **466** 154
[16] Denizot F and Lang R 1986 *J. Immunol. Methods* **89** 271
[17] Mehnert W and Mäder K 2012 *Advan. Drug Deliv. Rev.* **64** 83
[18] Nalwa HS 2004 *Encyclopedia of Nanoscience and Nanotechnology* **10** 43
[19] Fan H, Liu G, Huang Y, Li Y and Xia Q 2014 *Appl. Surf. Sci.* **288** 193
[20] Tamjidi F, Shahedi M, Varshosaz J and Nasirpour A 2013 *Innovative Food Sci. Emerging Technol.* **19** 29
[21] Baughman BM, Stennett E, Lipner RE, Rudawsky AC and Schmidtke SJ 2009 *J. Phys. Chem. A* **113** 8011
[22] Badawi AA, Elbayoomy TS, Abdelmalak NS and Sanad RA 2010 *Amer. Assoc. Pharm. Sci.* **11** 1684
[23] Lacerda SP, Cerize NNP and Ré MI 2011 *Inter. J. Cosmetic Sci.* **33** 312
[24] Rincón MAD 2011 *Ph.D. Thesis*, Chemical Engineering Faculty, UNICAMP, Campinas,Brazil