International Journal of Pharmaceutics 420 (2011) 133–140

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

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

Pharmaceutical Nanotechnology

Production and characterization of antioxidant apigenin nanocrystals as a novel UV skin protective formulation

Loaye Al Shaal, Ranjita Shegokar, Rainer H. Müller*

Freie Universität Berlin, Institute of Pharmacy, Department of Pharmaceutics, Biopharmaceutics & NutriCosmetics, Kelchstr. 31, 12169 Berlin, Germany

ARTICLE INFO

Article history:
Received 11 April 2011
Received in revised form 8 August 2011
Accepted 10 August 2011
Available online 17 August 2011

Keywords:
Apigenin
smartCrystals
Antioxidant activity
DPPH assay

ABSTRACT

Flavonoids have many positive effects on various cell layers of the skin (e.g. anti-oxidant, anti-allergic and anti-inflammatory effects). However, the limiting factor of the use of flavonoids is their low water solubility. To overcome the poor solubility, apigenin nanosuspensions were prepared using the combination technology (CT), i.e. bead milling and subsequently high pressure homogenization. Distinct reduction in particle size was observed after each bead milling passage resulting in *z*-average (PCS) of 413 nm and a polydispersity index of 0.202. The LD data showed a similar pattern of particle size reduction reaching a diameter 99% ($d(v)99\%$) of 0.515 μm. The antioxidant capacity of apigenin nanocrystals were almost doubled compared to the original coarse suspension. The developed smartCrystals can be easily incorporated into gels, which makes apigenin nanocrystals available for dermal application as efficient antioxidant.

1. Introduction

In the last two decades, the aging theories were thoroughly studied. As early as 1956, D. Harman proposed that free radicals cause cumulative oxidative damage to cells, causing aging and subsequently death of cells (Harman, 2006). The skin – the largest organ and an important protective barrier between the environment and the inner milieu – is a highly exposed tissue to oxidative stress in the body. It undergoes oxidative stress from both exogenous and endogenous sources (Kohen and Nyska, 2002; Masaki, 2010; Portugal et al., 2007). Exogenous sources contain environmental factors such as air pollutants, ionizing and non-ionizing radiation (Kohen and Nyska, 2002; Schabeuter and Wood, 1989), while endogenous sources consist of reactive species formed from activated phagocytes and enzymes which produce active oxygen metabolites (Rieger and Pains, 1993). Imbalance between antioxidation and oxidation may lead to oxidative stress involved in aging and various skin diseases (Rittie and Fisher, 2002). However, orally and dermally administered natural antioxidants such as superoxide dismutase (SOD), ferulic acid and flavonoids can protect the skin against the oxidative activity of UV irradiation (Graf, 1992; Montenegro et al., 1995). Although the phenolic nature of flavonoids gives them considerably high polarity but at the same time they exhibit poor solubility in water, thus the flavonoids have limited skin/dermal but also oral absorption (Havsteen, 2002).

Phytochemicals (e.g. phenolic acids, glucosinolates and flavonoids) are important nutrients produced by plants as secondary metabolites, especially when plants are in stress conditions, and may play a critical role in prevention of age-related diseases or even cancer (Ndiaye et al., 2011; Stevenson and Hurst, 2007). Innovative dosage forms were chosen to increase the bioavailability of these compounds and hence enhance their oral and dermal bioavailability such as biodegradable nanoparticles (Bala et al., 2005), nanoemulsions (Fasolo et al., 2009), liposomes (Gabrielska et al., 1997; Goniotaki et al., 2004; Naumov et al., 2010), phytosomes (Patela et al., 2009) and nanosuspensions (Mauludin et al., 2009; Mishra et al., 2009). Often the antioxidant potential of either extracts with herbal actives or the pure isolated actives were evaluated, but very few reports are available on the anti-oxidation properties of final formulations. Many of these phytochemicals e.g. quercetin, rutin have low water solubility which hinders their absorption, i.e. bioavailability (Holst and Williamson, 2008). Quite a number of flavonoid nanocrystals (e.g. rutin (Müller et al., 2007), hesperidin (Kobierski et al., 2008b), resveratrol (Kobierski et al., 2009) and hesperetin (Mishra et al., 2009)) were successfully produced for dermal application, but very limited data is available on the antioxidant activity of these nanocrystals (Petersen, 2006b; Wissing and Müller, 2002). Especially it would be interesting to assess to which extend the antioxidant effect increases after nanonization.

To overcome the solubility related problems, various approaches have been studied like the usage of surfactants (e.g. solubilisates, microemulsions), co-solvents and complexation with cyclodextrins (Kim et al., 2008). Most of these approaches may cause severe side effects ranging from skin irritation (e.g.

* Corresponding author. Tel.: +49 30 838 50696; fax: +49 30 838 50616.
E-mail address: nanoteam@gmx.com (R.H. Müller).

0378-5173/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2011.08.018

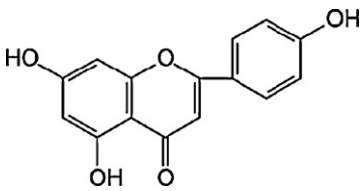

Fig. 1. Structure of apigenin (5,7-dihydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one).

surfactants, microemulsions) in case of systemic application (e.g. i.v.) – to nephrotoxicity, in case of cyclodextrins (Willems et al., 2001). Conversion of the microsized drug particles into nanocrystals (pure drug only stabilized with a small amount of stabilizer) not only increases the dissolution velocity (Buckton and Beezer, 1992; Hecq et al., 2005; Jinno et al., 2006; Keck, 2008; Müller et al., 2000), but also increases the saturation solubility, which significantly improves the bioavailability of the drug. An increase in the saturation solubility increases the concentration gradient between the dermally applied formulation and the skin (Mishra et al., 2009). In addition, prolonged contact after dermal application can even be achieved due to the adhesive properties of nanomaterials (Müller and Jacobs, 2002).

Nanocrystals can be produced by the "bottom-up" (e.g. Nanomorph™, Soliqs/Abbott, PCT/EP00/0635), "top-down" technologies (e.g. Dissocubes®, SkyePharma, US Patent 5,858,410) or combination technologies (e.g. NANOEDGE, Baxter (Kipp et al., 2003)) Nanocrystals in pharmaceutical and cosmetic products or in clinical trials are based on top-down technologies.

Top-down technologies provide a relatively easy scale up opportunity and are therefore better suited for industry. The nanocrystals are produced by wet milling methods, e.g. bead milling [low energy milling technology (Nanosystems/élan (Liversidge, 2003)] or high pressure homogenization [Dissocubes® technology (Müller et al., 2008)]. In 2001, a second generation of drug nanocrystals (smartCrystals®) was introduced based on various combinations of technologies with improved characteristics (e.g. physical stability) and the option for lower particle size. The smartCrystal technology is a toolbox of various processes, providing nanocrystals optimized for the respective drug and application route (e.g. processes H69 (Müller and Möschwitzer, 2007), H96 (Möschwitzer and Lemke, 2007) and the combination technology (CT) (Petersen, 2006b). In the CT process, the crystals are firstly processed by pearl milling, and subsequently passed through a high pressure homogenizer (Keck et al., 2008; Petersen, 2006b). This results in nanosuspensions with higher physical stability, compared to bead milling alone, being of interest for dermal formulations which can contain destabilizing electrolyte excipients or actives (e.g. preservative, salts, dissociated drugs or cosmetic actives). In this study smartCrystals® were chosen as an innovative dosage form to formulate poorly water-soluble drugs. In contrast to other formulations such as liposomes, solid lipid nanoparticles (SLN) and nanocomplexes, the nanocrystal increases the kinetic saturation solubility of the actives. In addition, they have the advantages of simplicity in production and their feasibility to be easily scaled-up (Al Shaal et al., 2010; Shegokar and Müller, 2010) as compared to the other carriers. This study was designed to investigate the effect of nanonization on the anti-oxidation potential of apigenin (Fig. 1) nanocrystals compared to their macrosuspension using the free radical 2,2-Diphenyl-1-picrylhydrazyl (DPPH•) method (Brandwilliams et al., 1995).

2. Materials and methods

2.1. Materials

Apigenin was purchased from Exquim, S.A. (Spain) and the stabilizer Plantacare 2000 UP® (alkyl polyglycoside) was provided by Cognis GmbH (Germany). As dispersion medium, freshly prepared double distilled and ultra purified water (Milli-Q, Millipore GmbH, Germany) was used. Sodium chloride solution (NaCl) 0.9% was purchased from B. Braun Melsungen AG (Germany).

2.2. Methods

2.2.1. Production of smartCrystals

Nanocrystals were produced using an agitated Bühler PML-2 bead/pearl mill (Bühler AG, Switzerland) in the discontinuous mode containing yttria stabilized zirkonia (0.4–0.6 mm) as milling medium. Apigenin macrosuspensions (5% w/w) were prepared by dispersing the powder in an aqueous surfactant solution (Plantacare 2000® UP, 1% w/w). Dispersing was performed by high shear mixing at 10,000 rpm using an Ultra-Turrax T25 (Janke and Kunkel GmbH, Germany) for 1 min, followed by bead milling applying seven passages through the Bühler PML-2. In the second subsequent step, the bead-milled nanosuspension was passed through an Avestin C50 (Avestin Europe GmbH, Germany) high pressure homogenizer at low pressure of 300 bar for one cycle to disperse the possible aggregates that may still exist after bead milling (Fig. 2).

2.2.2. Characterization of nanosuspensions

2.2.2.1. Photon correlation spectroscopy (PCS). PCS was performed using a Zetasizer Nano ZS (Malvern Instruments, UK). The analysis yields the *z*-average (intensity weighted mean diameter of the bulk population) and the polydispersity index (a measure of the width of the size distribution, PdI). The nanosuspension samples were diluted in distilled water, 10 measurements were performed for each sample at 25 °C and a mean was reported.

2.2.2.2. Laser diffractometry (LD). Laser diffraction (static light scattering) was performed using a Mastersizer 2000 (Malvern Instruments, UK) in deionized water as dispersion medium using the Hydro S sample dispersion unit. The LD volume weighted diameters $d(v)50\%$, $d(v)90\%$, $d(v)95\%$ and $d(v)99\%$ were used as characterization parameters. All parameters have been analyzed using the Mie theory with 1.730 and 0.01 for the real refractive index and imaginary refractive index, respectively. The real refractive index was calculated from measured refractive indices of apigenin solutions and extrapolating to 100% apigenin.

2.2.2.3. Light microscopy. Light microscopy (Orthoplan, Leitz, Germany) was performed on samples obtained after production to detect the presence of potential larger particles (>1 μm). Polarized light was also used to confirm the presence or absence of larger crystals or even aggregates using magnification of 160-fold and 1000-fold.

2.2.2.4. Zeta potential (ZP) measurements. The surface charge of the particles was assessed by zeta potential measurements using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK) at 25 °C. The measured electrophoretic mobility was converted to the zeta potential by the Helmholtz–Smoluchowski equation. The zeta potential was determined in conductivity adjusted Milli-Q water (50 μS/cm, using 0.9% NaCl solution) and in the original surfactant solution.

2.2.2.5. X-ray diffraction studies (XRD). XRD was performed at room temperature with a Philips X-ray Generator PW 1830 (Philips, The Netherlands) for bulk powder and the aqueous nanosuspension after drying. The diffraction angle range was between 0.6° and 40° with a step size of 0.04° per 2 s. The diffraction pattern for each sample was measured at a voltage of 40 kV and a current of 25 mA.

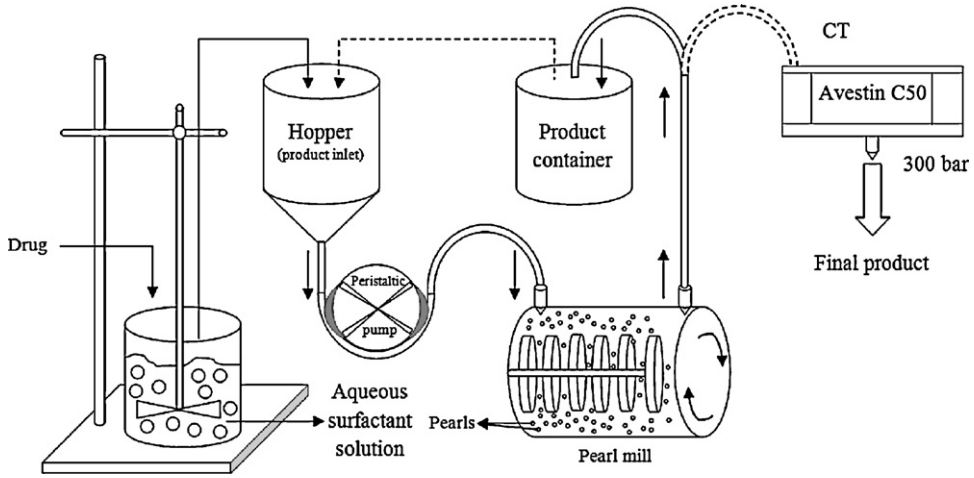

Fig. 2. Schematic diagram for production of apigenin smartCrystals.

2.2.2.6. Determination of free radical scavenging activity. The antiradical activity of coarse and nanonized apigenin was measured according to the procedure described by Brandwilliams and colleagues. Initially, a set of increased concentrations of DPPH• in methanol (0.01–0.3 mM) was prepared in order to get the standard curve. The medium concentration was scanned for the maximum absorbance at different wavelengths using UV–Vis spectrophotometer (UV-1700 PharmaSpec, Schimadzu, Japan). Definite amounts of the nanosuspension (0.1 ml of the 5% apigenin nanocrystals) were mixed with 3.9 ml DPPH• methanolic solution (0.025 g/100 ml). Absorbance was measured (λ_{max} = 254 nm) at different time intervals until the reaction reached a plateau. The time required to reach plateau was further defined as the steady state time (T_{ss}). The percentages of residual DPPH• (n = 3) were plotted against the ratios of the antioxidant to the DPPH•. Efficient concentration required to decrease the DPPH• by 50% (EC_{50}) was then calculated from the previously obtained standard curve equation. Ascorbic acid was selected as a standard.

3. Results and discussion

3.1. Production of aqueous nanosuspensions of apigenin

The smartCrystal technology is actually a family of different combination processes, a pre-treatment step followed by a high pressure homogenization (HPH) step. The aims are e.g. to accelerate nanocrystals production (less homogenization cycles) or to obtain smaller nanocrystals sizes. For example, the H96 process consists of lyophilization of an organic solution of the poorly water soluble drug followed by homogenizing in aqueous phase (Möschwitzer and Lemke, 2007). Lyophilization makes the drug material more fragile, and nanocrystals < 100 nm are obtained, a size range normally not accessible by HPH alone. In the CT process, the milling is followed by HPH (Petersen, 2006b). This process has the advantage, that more viscous macrosuspension with higher drug concentrations can be processed as compared to a homogenizer, as a rule of thumb 2–3 times higher. In addition, nanosuspensions treated in the second step by HPH possess a higher electrolyte stability and physical long-term stability.

For these reasons the smartCrystal CT process was applied in this study for production of apigenin nanocrystals. The macrosuspension was passed through the mill 7 times to achieve the maximum dispersitivity (=smallest particle size achievable at the applied milling conditions, e.g. pearl size, energy input). A batch size of 1 kg was produced, but the set up remains the same even for producing a 100 kg batch. It requires only longer milling times. A continuous particle size reduction was observed after each passage until the 5th passage, no relevant, very little further reduction occurred after the 6th and 7th passage. Therefore bead milling was terminated after 7 passages. Further particle size reduction of the obtained nanosuspension was observed by passing it through the homogenizer at a pressure of 300 bar. Apigenin nanosuspension reached a PCS diameter of 396 ± 12 nm with a PdI of 0.205 ± 0.007 and an LD diameter $d(v)$99% 0.547 ± 0.003 μm (Fig. 3). It can be clearly seen from these results that the particle size of nanosuspension was well below 1 μm and it has a reasonably low PdI. This indicates a successful production and optimum production parameters of nanosuspensions. The obtained crystal size depends not only on the hardness of the drug, but also the size of the milling beads used. As a rule of thumb, the particle size is approximately 1000 fold smaller than the bead size used. The bead size was 0.4–0.6 mm, the bead size about 0.4 μm hence 400 nm particle size), results obtained confirm this rule.

3.2. Characterization of nanocrystals

3.2.1. Particle size analysis

In the smartCrystal CT process, the bead milling is a low energy process–in contrast to the high energy process HPH. After the first passage the PCS diameter is about 1543 ± 73 nm. Applying a second passage leads to a very distinct decrease to about 830 ± 79 nm, followed by a slowly decrease until completion of passage 5. This is the smallest size, further passages until 7 have practically no effect on the size, and little effect on the PdI. After 7 passages, the mean particle size was 439 ± 20 nm with a low polydispersity index of 0.283 ± 0.040 (Fig. 3). A sharp decline in the polydispersity index was observed from 0.588 to 0.265 from the 1st passage to the 7th passage.

Passing bead-milled nanosuspensions in a subsequent homogenization step at low pressures (e.g. 100–500 bar) yielded smaller and more homogenous nanocrystal populations than when applying high pressures, e.g. 1000–1500 bar (Petersen, 2006b). When the size distribution gets narrower, the PdI decreases with a significant improvement in the physical stability. However, pressures below 300 bar could not be precisely adjusted. This is the reason, why 300 bar were applied in this study. With some drugs, a further decrease in size was observed in the final HPH step, with others the size stayed unchanged and only the PdI decreased. In the case of apigenin the mean PCS particle size decreased slightly by about 10% (decrease from 439 nm after bead milling to 396 nm), further slight decrease in polydispersity index was observed.

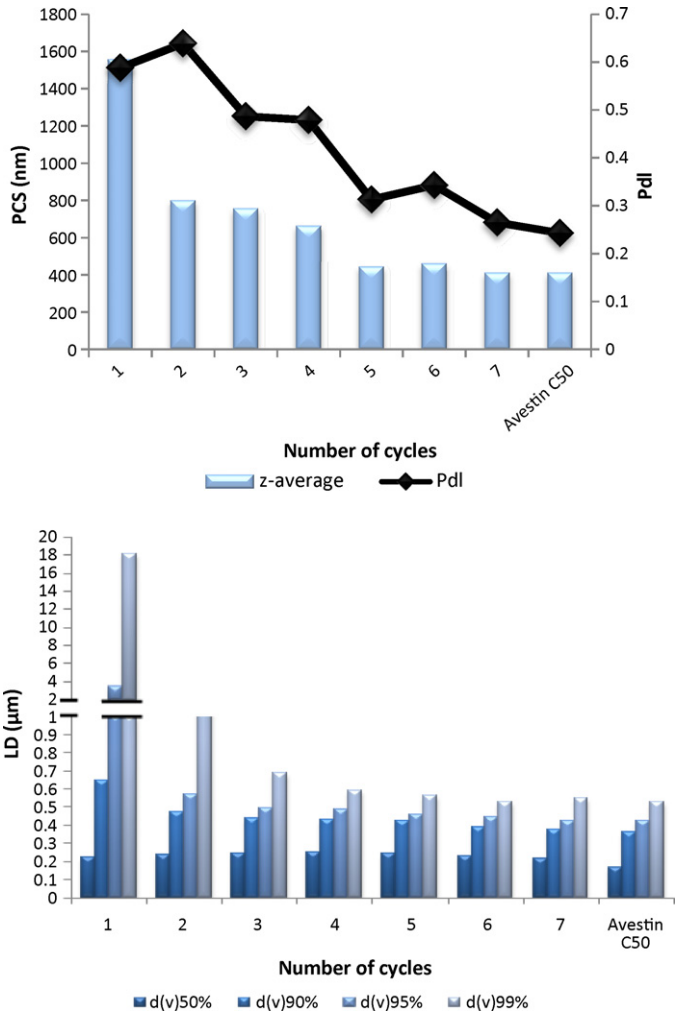

Fig. 3. PCS diameters (*z*-average) and polydispersity indices (PdI) (upper) and LD diameters 50%, 90%, 95% and 99% of apigenin nanosuspensions (lower) produced on pilot scale as function of passages through the bead mill (1–7) and after final passage through the Avestin C50, 1 cycle at 300 bar on day of production.

Data obtained from LD showed that the $d(v)95\%$ and $d(v)99\%$ decreased continuously till the 5th passage, but further increase in the number of passages to 7 did not show any changes. This is in agreement with the PCS data, that minimum dispersivity for the bulk population has been reached after 5 passages. The content of remaining particles larger than the bulk population (quantified by $d(v)95\%$ and $d(v)99\%$) reaches also the minimum after 5 passages. This is in contrast to other drugs, for which further reduction of remaining larger particles was found applying additional passages after reaching the smallest size of the bulk population.

The LD diameter 50% of the raw material was about 9.297 μm, which dropped sharply to around 0.220 μm after the 1st passage through the bead mill. Then it remained unchanged till the 7th passage, i.e. 0.231 ± 0.018 μm. In contrast, the PCS measurements revealed a slight but steady decrease in the size of bulk population. This shows that the LD is less sensitive in detecting smaller changes in the bulk population. It can be explained by the differences in the measuring ranges of the two methods, being approx. 3 nm to 3 μm/6 μm for PCS but 40 nm to 2000 μm for LD. Consequently smaller changes in the low nanometer range are not that well detectable by LD, comparable to measure sizes below 1 mm when using a caliper versus an inch rule with a 2 m measuring range.

However, a further reduction in $d(v)50\%$ to 0.164 ± 0.015 μm was obtained after passing milled nanosuspension through Avestin C50, which is in accordance to the PCS data (reduction by 10%) and the published theory (Petersen, 2006b).

3.2.2. Light microscopy

Light microscopy is a frequently used technique for the detection of presence or absence of large crystals or aggregates (>1 μm) in nanosuspensions. Aim was not to measure the size of the nanocrystals (being close to or even below the resolution of the microscope) but to detect few large particles which might not be detectable by LD. Light microscopy serves as a complementary sizing method to LD. Samples were investigated undiluted to increase the probability to find even a few large particles (Müller and Heinemann, 1993).

Fig. 4 shows light microscopic pictures of apigenin smartCrystals on day of production after the 7th passage through the bead mill (A), after treatment by HPH at 300 bar for one cycle (B) and the same sample under polarized light (C) (160 fold magnification). A uniform crystal distribution with absence of large crystals and aggregates was seen for the nanosuspension both after HPH and after bead milling. This is well in agreement with the previously published finding that Plantacare 2000 at 1% concentration provides optimal stability for apigenin nanosuspensions (Kerč et al., 2009).

3.2.3. Zeta potential measurements

The charge of the particles, expressed as zeta potential, is an important stability determining parameter. Measurements were performed in distilled water (for measuring the Stern potential) and in the original dispersion medium of the suspension (for measuring the thickness of the diffuse layer). The Stern potential is related to the Nernst potential, and therefore an indirect measure of the charges on the nanocrystal surface. The higher is the charge, the better is the physical stability. Data obtained from measurement conducted in distilled water showed a ZP of −38 mV, indicating a well charged surface and related stability. The higher the zeta potential measured in the original dispersion medium, the thicker is the diffuse layer and the more stable is the suspension. Plantacare 2000 possesses a ZP of about −37 mV in the original dispersion medium. This is well above the critical 30 mV (Riddick, 1968) and indicates a good physical stability during storage. Stability is even higher than estimated from the −37 mV because Plantacare® 2000 has additionally a steric stabilizing effect. The apigenin nanosuspension possesses good electrostatic plus some steric stabilization, explaining the previously observed long-term stability of this formulation composition (Kerč et al., 2009; Kobierski et al., 2008a).

3.2.4. X-ray diffraction studies (XRD)

Fig. 5 shows X-ray diffraction patterns of apigenin coarse powder and dried smartCrystals. X-ray diffraction pattern of both coarse powder and dried nanocrystals show highly crystalline form, no decreased intensities were observed in case of nanocrystals. Milling in combination with HPH technology was efficient in obtaining physically stable crystalline nanocrystals of apigenin, avoiding the formation of an undesired amorphous fraction (within the detection limit of the method of course). Amorphous fractions or polymorphic modifications can be formed when applying high pressures as known from tabletting (Nyström, 1998), However, this does not be to seem an issue for homogenized nanosuspensions. In our previous studies with anti-oxidant rutin nanocrystals we observed also practically identical diffraction patterns for coarse powder and prepared nanocrystals. In 20 years of history in our research group preparing nanosuspensions by high pressure homogenization only with one drug conversion to the amorphous state took place. Amorphous drug nanoparticles possess even

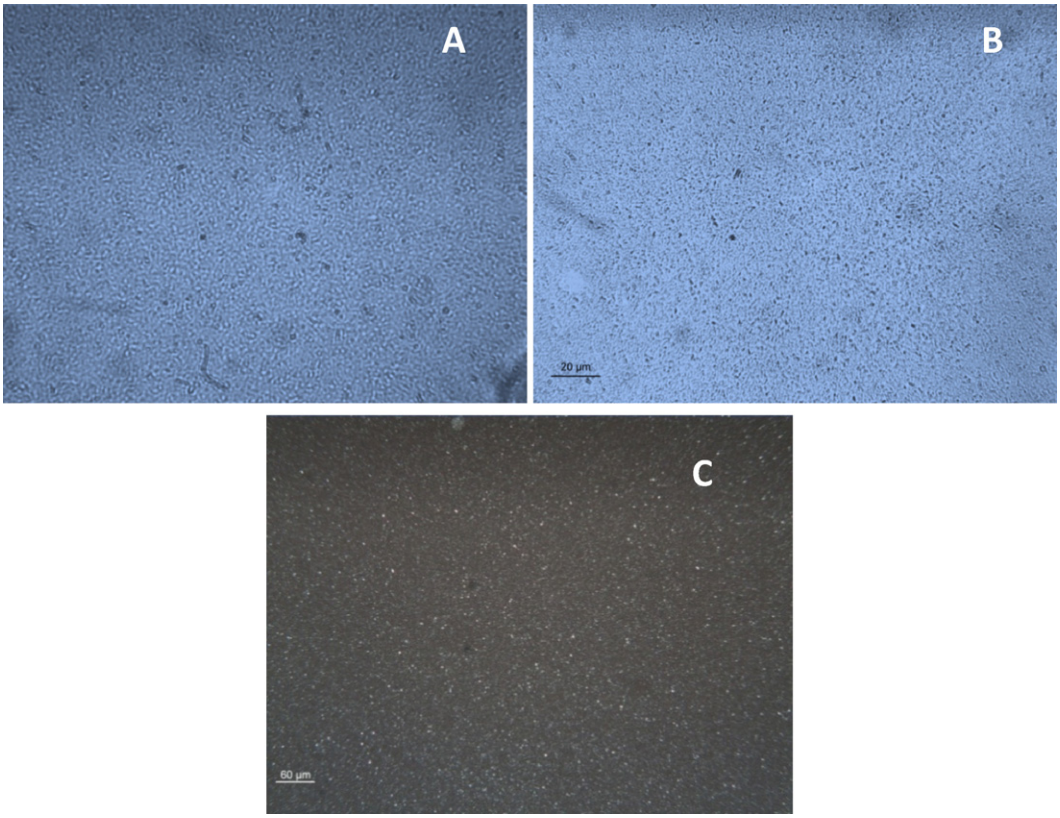

Fig. 4. Light microscopic pictures of apigenin smartCrystals on day of production after the 7th passage of bead milling (A), after treatment by HPH at 300 bar applying one cycle (B), and under polarized light (C) (160 magnification).

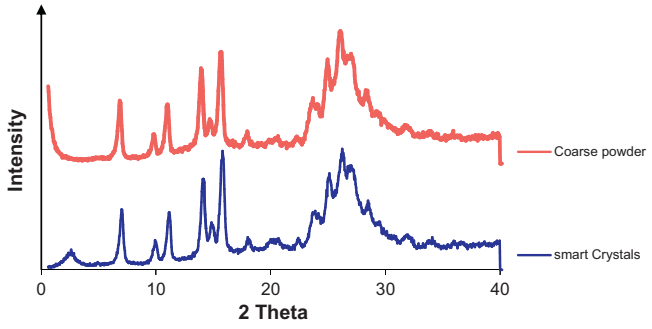

Fig. 5. X-ray diffractograms of the apigenin coarse powder and the dried smartCrystals.

better solubility properties than nanocrystals, but they bear the risk of conversion to the crystalline state during the shelf life of a product. Thus, industry avoids them when the delivery problem can be solved using crystalline drug nanoparticles, i.e. nanocrystals.

3.2.5. Determination of the antiradical activity

The DPPH• radical scavenging test is often reported in literature and is a very convenient method for screening antioxidant molecules as one can see the reaction visually (Antolovich et al., 2002). The detection can be simply carried out using a UV–Vis spectrophotometer. The DPPH• radical is scavenged by the antioxidants through the donation of hydrogen to form the stable reduced DPPH-H molecule. The antiradical activity can be analyzed by plotting the residual percentage of DPPH• – after reaching the steady state – against the molar ratios of the antioxidant used to DPPH•.

In general, antioxidant activity depends on structural features such as O–H bound dissociation energy, resonance delocalization of phenol radicals and steric hindrance derived from bulky groups substituting hydrogen in the aromatic ring (Fig. 6) (Shahidi and Naczk, 1995). The concentration and the structure of the antioxidant play a crucial role in the residual amount of DPPH•.

A calibration curve of DPPH• in methanol was measured in concentrations of 10 to 300 µM. λ_{max} was noted at 515 nm. For the calculation of the DPPH• concentrations in further experiments a calibration curve equation was used (Eq. (1)).

$$y = 12.612x(\text{g/l}) \quad r^2 = 0.9997 \tag{1}$$

The time required to achieve the steady state (T_{ss}) was determined in order to ensure the endpoint of reaction. For apigenin nanocrystals the T_{ss} was reached after 330 min (Fig. 7). This can be due to the low solubility of apigenin in the methanol solution of DPPH•, which leads to slow reaction between the apigenin molecules and the DPPH• ones. This results from the fact that the reaction takes place only on the surface of particles and between the slow amount of dissolved apigenin molecules and DPPH•.

The EC_{50} (efficient concentration required to decrease the DPPH• concentration by 50%) of apigenin macro- and nanosuspensions was calculated by plotting the residual percentage of DPPH• against the molar ratios of the antioxidant to DPPH• (Fig. 8). The lower the EC_{50}, the higher is the antioxidant activity of a compound. EC_{50} value of apigenin macro- and nanosuspensions were 4.583 ± 0.164 and 2.993 ± 0.098, respectively. The nanosuspension possessed almost 2 times higher than the antioxidant activity of the macrosuspension (Fig. 9).

The effect of nanonization can be clearly seen. The particle size in the nanometer range has a higher antioxidation potential compared to the micron-sized coarse suspension. More surface area is available for the DPPH reaction. In addition, the nanosuspension has higher saturation solubility. The solubility of the macrosuspension and the nanosuspension in the reaction medium were 1072

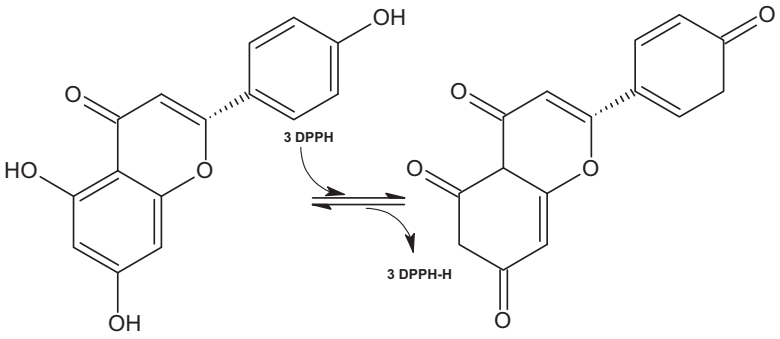

Fig. 6. Reaction mechanism of DPPH and apigenin.

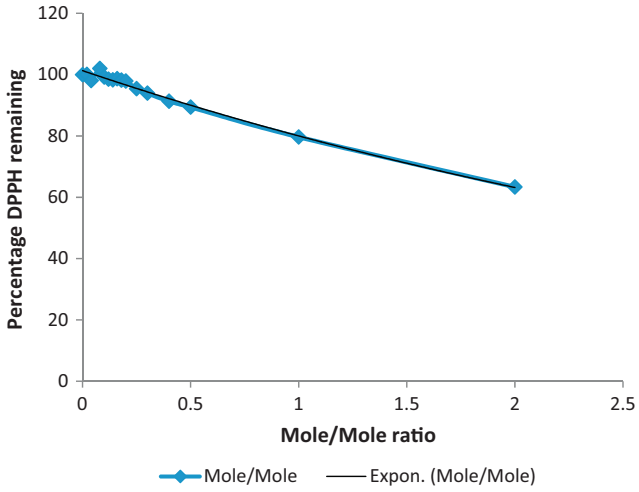

Fig. 7. Declining percentage of DPPH versus the molar ratios of the antioxidant used to DPPH•.

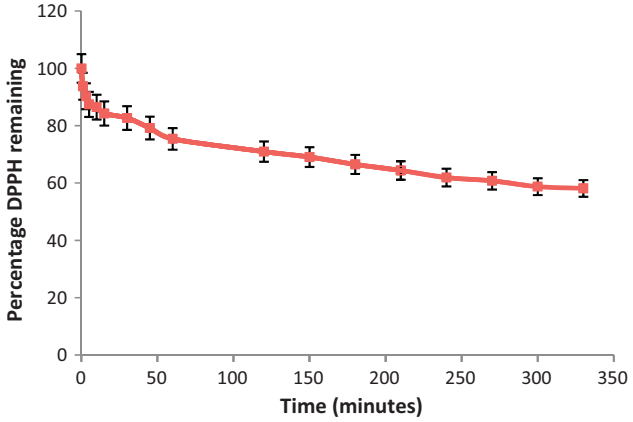

Fig. 8. Declining percentage of DPPH and reaching the steady state at 330 min for the antioxidant reaction of apigenin and DPPH.

and 1204 μg/ml, respectively, i.e. the saturation solubility of the nanosuspension was only slightly higher. The increased saturation solubility in methanol and primarily the surface area augmentation play a determining role in the increased antioxidant activity of apigenin nanosuspension.

Both macro- and nanosuspensions were compared to the antioxidant activity of ascorbic acid. Ascorbic acid reacted rapidly with DPPH• reaching a steady state after 1 min unlike apigenin with a slow kinetic behavior of 330 min. The EC_{50} of ascorbic acid (two

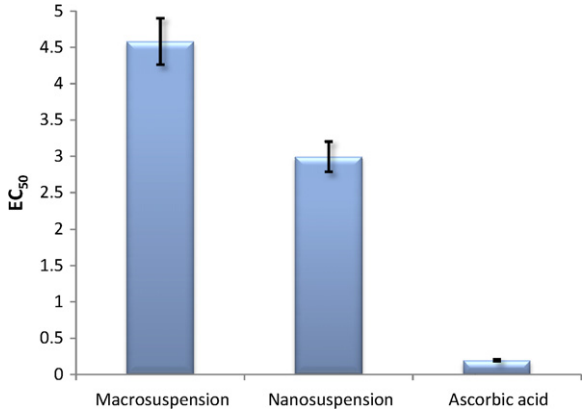

Fig. 9. EC50 of apigenin macro- and nanosuspension in comparison with standard (ascorbic acid).

hydroxyl group) reached 0.192, showing very high efficacy as an antioxidant, with a fast kinetic behavior as compared with apigenin (three hydroxyl group) nanosuspension with value of 2.99, as the difference in the antioxidant activity is highly dependent on the number of available hydroxyl groups. However, the ascorbic acid as hydrophilic molecule has lower skin permeability than apigenin. Therefore despite higher EC_{50} the apigenin might have better efficiency in the skin due to its better penetration ability, similar to poorly soluble lipophilic rutin versus hydrophilic rutin-glucoside (cf. 3.2.6)

This experiment proves that nanosuspensions can significantly enhance the antioxidation potential for poorly soluble compounds. It was found that also the nanosuspension of resveratrol has a better antioxidation activity than the normal powder (unpublished data). To summarize, nanonization increase the surface area which is available for dissolution and simultaneously enhancement in antioxidation potential. Nanonization approach can be used for poorly synthetic and herbal actives to enhance their biological effects for various applications especially for oral and dermal use.

3.2.6. Release profile and permeability

Due to their large surface area and increased saturation solubility, the nanocrystals dissolve very fast, typically within 5 min at sink conditions in the USP paddle method (Shegokar and Müller, 2010). In a nanocrystal-containing cream, the active is dissolved in the water phase at its kinetic saturation solubility, providing an enhanced concentration gradient between dermal formulation and skin compared to "μm" sized powders. This leads to increased penetration, being exploited for example for rutin and hesperidin nanocrystals (Petersen, 2006a). In a human *in vivo* study the antioxidant capacity of both molecules was assessed by measuring the sun protection factor (SPF). For example, for the rutin

nanocrystal cream the SPF increased 2 times as high at only 1/500 of dissolved molecules in the cream compared to a water soluble rutin-glucoside derivative, i.e. increase in bioactivity by a factor $2 \times 500 = 1000$. This was not only attributed to the increased concentration gradient, but also to the better skin permeability of the original molecule compared to the water soluble derivative, which just liked to stay in the hydrophilic environment of the cream than penetrating through lipophilic parts of the skin. The same dissolution and permeability properties apply to the apigenin nanocrystals.

4. Conclusions

It could be shown that second generation technology of nanosuspensions (smartCrystals) is very effective for formulation of poorly soluble apigenin, i.e. in this case the CT process. Especially the second step of homogenization yields a smaller sized more homogenous product, favoring physical stability (avoidance/minimization of Ostwald ripening). The crystallinity is remained, avoiding problems with amorphous fractions.

It could be shown that the *in vitro* antioxidant activity almost doubled by creating a nanosuspension. This makes this formulation interesting for dermal application. The obtained *in vitro* results are well in agreement with *in vivo* data of a human study with antioxidant rutin nanocrystals (Müller et al., 2007; Petersen, 2006b). In this study the increase in SPF as measure for the antioxidant activity in the skin was double for the rutin nanocrystals compared to a water soluble rutin derivative. The CT process is suitable for large scale, opening the option to use these apigenin nanocrystals in cosmetic anti-aging products or pharmaceutical creams for supportive skin cancer protection.

References

Al Shaal, L., Müller, R.H., Shegokar, R., 2010. smartCrystal combination technology—scale up from lab to pilot scale and long term stability. Pharmazie 65, 877–884.

Antolovich, M., Prenzler, P.D., Patsalides, E., McDonald, S., Robards, K., 2002. Methods for testing antioxidant activity. Analyst 127, 183–198.

Bala, I., Bhardwaj, V., Hariharan, S., Sitterberg, J., Bakowsky, U., Kumar, M.N.V.R., 2005. Design of biodegradable nanoparticles: a novel approach to encapsulating poorly soluble phytochemical ellagic acid. Nanotechnology 16, 2819–2822.

Brandwilliams, W., Cuvelier, M.E., Berset, C., 1995. Use of a free-radical method to evaluate antioxidant activity. Food Sci. Technol.—LWT 28, 25–30.

Buckton, G., Beezer, A.E., 1992. The relationship between particle size and solubility. Int. J. Pharm. 82, R7–R10.

Fasolo, D., Bassani, V.L., Teixeira, H.F., 2009. Development of topical nanoemulsions containing quercetin and 3-O-methylquercetin. Pharmazie 64, 726–730.

Gabrielska, J., Oszmianski, J., Zylka, R., Komorowska, M., 1997. Antioxidant activity of flavones from *Scutellaria baicalensis* in lecithin liposomes. Z. Naturforsch. C—J. Biosci. 52, 817–823.

Goniotaki, M., Hatziantoniou, S., Dimas, K., Wagner, M., Demetzos, C., 2004. Encapsulation of naturally occurring flavonoids into liposomes: physicochemical properties and biological activity against human cancer cell lines. J. Pharm. Pharmacol. 56, 1217–1224.

Graf, E., 1992. Antioxidant potential of ferulic acid. Free Radic. Biol. Med. 13, 435–448.

Harman, D., 2006. Free radical theory of aging: an update: increasing the functional life span. Ann. N. Y. Acad. Sci. 1067, 10–21.

Havsteen, B.H., 2002. The biochemistry and medical significance of the flavonoids. Pharmacol. Therapeut. 96, 67–202.

Hecq, J., Deleers, M., Fanara, D., Vranckx, H., Amighi, K., 2005. Preparation and characterization of nanocrystals for solubility and dissolution rate enhancement of nifedipine. Int. J. Pharm. 299, 167–177.

Holst, B., Williamson, G., 2008. Nutrients and phytochemicals: from bioavailability to bioefficacy beyond antioxidants. Curr. Opin. Biotechnol. 19, 73–82.

Jinno, J., Kamada, N., Miyake, M., Yamada, K., Mukai, T., Odomi, M., Toguchi, H., Liversidge, G.G., Higaki, K., Kimura, T., 2006. Effect of particle size reduction on dissolution and oral absorption of a poorly water-soluble drug, cilostazol, in beagle dogs. J. Control. Release 111, 56–64.

Keck, C.M., 2008. NanoCrystal® Technology: a formulation approach for poorly water soluble compounds. In: Sciences, I.L. (Ed.), Particle Design for APIs and Drug Products. 8–10th September, Brussels.

Keck, C.M., Kobierski, S., Mauludin, R., Müller, R.H., 2008. Second generation of drug nanocrystals for delivery of poorly soluble drugs: smartCrystals technology. Dosis 2, 124–128.

Kerč, J., Kobierski, S., Keck, C.M., Müller, R.H., 2009. Influence of different stabilizers on particle size of apigenin nanosuspensions produced by high pressure homogenization. International Symposium on Controlled Release of Bioactive Materials, vol. 36. Controlled Release Society, Inc., Copenhagen, p. 3885.

Kim, D.J., Jang, H.D., Kim, E.J., Koo, K.K., 2008. Effect of reaction temperatures and media on crystal structure of colloidal nanocrystals synthesized from an aerosol flow system. Ultramicroscopy 108, 1278–1282.

Kipp, J.E., Wong, J.C.T., Doty, M.J., Rebbeck, C.L., 2003. Microprecipitation method for preparing submicron suspensions. In: United States Patent 6,607,784. Baxter International Inc., Deerfield, IL, USA.

Kobierski, S., Al Shaal, L., Keck, C.M., Kerč, J., Müller, R.H., 2008a. Preparation and physical evaluation of ascorbyl palmitate nanosuspensions. In: AAPS Annual Meeting, Atlanta, USA.

Kobierski, S., Mauludin, R., Keck, C.M., Müller, R.H., 2008b. Production of Hesperidin dermal nanocrystals by novel smartCrystal combination technology. In: European Workshop on Particulate Systems, 30–31th May, Berlin.

Kobierski, S., Ofori-Kwakye, K., Muller, R.H., Keck, C.M., 2009. Resveratrol nanosuspensions for dermal application—production, characterization, and physical stability. Pharmazie 64, 741–747.

Kohen, R., Nyska, A., 2002. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol. Pathol. 30, 620–650.

Liversidge, G., 2003. Workshop Nanotechnology—solid particles, lipids and nanocomplexes. In: IIR Drug Delivery Partnerships™ Meeting, Cologne, Germany.

Masaki, H., 2010. Role of antioxidants in the skin: anti-aging effects. J. Dermatol. Sci. 58, 85–90.

Mauludin, R., Müller, R.H., Keck, C.M., 2009. Development of an oral rutin nanocrystal formulation. Int. J. Pharm. 370, 202–209.

Mishra, P.R., Al Shaal, L., Müller, R.H., Keck, C.M., 2009. Production and characterization of Hesperetin nanosuspensions for dermal delivery. Int. J. Pharm. 371, 182–189.

Montenegro, L., Bonina, F., Rigano, L., Giogilli, S., Sirigu, S., 1995. Protective effect evaluation of free radical scavengers on UVB induced human cutaneous erythema by skin reflectance spectrophotometry. Int. J. Cosmetic Sci. 17, 91–103.

Möschwitzer, J., Lemke, A., 2007. Method for carefully producing ultrafine particle suspensions and ultrafine particles and use thereof. In: Property, W.I. (Ed.), PCT/EP2006/003377. ABBOTT GmbH & CO.KG, Germany.

Müller, R.H., Hanisch, J., Mauludin, R., Petersen, R., Keck, C.M., 2007. Rutin drug nanocrystals for dermal cosmetic application. In: AAPS Annual Meeting.

Müller, R.H., Heinemann, S., 1993. Emulsions for Intravenous Application. II. Destabilisation and stabilisation mechanisms in fat emulsions. Die Pharm. Ind. 55, 948–953.

Müller, R.H., Jacobs, C., 2002. Buparvaquone mucoadhesive nanosuspension: preparation, optimisation and long-term stability. Int. J. Pharm. 237, 151–161.

Müller, R.H., Jacobs, C., Kayser, O., 2000. Nanosuspensions for the formulation of poorly soluble drugs. In: Nielloud, F., Marti-Mestres, G. (Eds.), Pharmaceutical Emulsions and Suspensions. Marcel Dekker, pp. 383–407.

Müller, R.H., Keck, C.M., Petersen, R.D., 2008. Rutin smartCrystals: bioactivity enhancement and stability against electrolytes. In: Annual Meeting of the American Association of Pharmaceutical Scientists (AAPS), Atlanta, USA.

Müller, R.H., Möschwitzer, J., 2007. Method and device for producing very fine particles and coating such particles. In: Property, W.I. (Ed.), PCT/EP2006/009930. ABBOTT GmbH & CO. KG, Germany.

Naumov, A.A., Shatalin, Y.V., Potselueva, M.M., 2010. Effects of a nanocomplex containing antioxidant lipid, and amino acid on thermal burn wound surface. Bull. Exp. Biol. Med. 149, 62–66.

Ndiaye, M., Kumar, R., Ahmad, N., 2011. Resveratrol in cancer management: where are we and where we go from here? Resveratrol Health 1215, 144–149.

Nyström, C., 1998. Dissolution properties of soluble drugs: theoretical background and possibilities to improve the dissolution behavior. In: Müller, R.H., Benita, S., Böhm, B. (Eds.), Medpharm Scientific. Stuttgart, pp. 143–147.

Patela, J., Patelb, R., Khambholjab, K., Patela, N., 2009. An overview of phytosomes as an advanced herbal drug delivery system. Asian J. Pharm. Sci. 4, 363–371.

Petersen, R., 2006a. Nanocrystals for use in topical cosmetic formulations and method of production thereof. US Patent 60/8866233.

Petersen, R.D., 2006b. Nanocrystals for use in topical formulations and method of production thereof. In: PCT/EP2007/009943. Abbott GmbH, Petersen, R.D, Germany.

Portugal, M., Barak, V., Ginsburg, I., Kohen, R., 2007. Interplay among oxidants, antioxidants, and cytokines in skin disorders: present status and future considerations. Biomed. Pharmacother. 61, 412–422.

Riddick, T.M., 1968. Control of colloid stability through zeta potential; with a closing chapter on its relationship to cardiovascular disease. Zeta-Meter, Inc, Wynnewood, PA.

Rieger, M.M., Pains, M., 1993. Oxidative reactions in and on the skin: mechanism and prevention. Cosmetic Toiletries 108, 43–56.

Rittie, L., Fisher, G.J., 2002. UV-light-induced signal cascades and skin aging. Ageing Res. Rev. 1, 705–720.

Schabeuter, K.U., Wood, J.M., 1989. Free radicals in the human epidermis. Free Radic. Biol. Med. 6, 519–532.

Shahidi, F., Naczk, M., 1995. Food phenolics: an overview. In: Shahidi, F., Naczk, M. (Eds.), Food Phenolics: Sources, Chemistry, Effects, Applications. Echnomic Publishing Company Inc, Lancaster, pp. 1–5.
Shegokar, R., Müller, R.H., 2010. Nanocrystals: Industrially feasible multifunctional formulation technology for poorly soluble actives. Int. J. Pharm. 399, 129–139.
Stevenson, D.E., Hurst, R.D., 2007. Polyphenolic phytochemicals—just antioxidants or much more? Cell. Mol. Life Sci. 64, 2900–2916.
Willems, L., van der Geest, R., de Beule, K., 2001. Itraconazole oral solution and intravenous formulations: a review of pharmacokinetics and pharmacodynamics. J. Clin. Pharm. Ther. 26, 159–169.
Wissing, S.A., Müller, R.H., 2002. The development of an improved carrier system for sunscreen formulations based on crystalline lipid nanoparticles. Int. J. Pharm. 242, 373–375.