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Azithromycin nanocrystals for dermal prevention of tick bite infections

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Azithromycin was optimized as nanocrystals with a drug content of 10.0 % (w/w) and a surfactant D- α -tocopheryl polyethylenglycol 1000 succinate (TPGS) content of 1.0 % (w/w) using bead milling for 10 min. The photon correlation spectroscopy (PCS) diameter of the bulk population was 189 nm, laser diffraction (LD) diameter 90 % was 370 nm. Spherical morphology of the optimal nanocrystals was observed by transmission electron microscope (TEM). They were stable over 1 year of storage at 4 °C with the particle size within the nanometer range which was confirmed by PCS, LD and light microscope. An acceptable physical stability of 2 years was also obtained when stored at 4 °C. No microbial attack to the nanocrystals was observed before 3 years storage at 4 °C. The saturation solubility of the nanocrystals was up to triple compared to the raw drug powder (RDP) in water. When incorporated into the gel base, highest penetration efficacy was achieved by the optimal nanocrystals compared to 1) the clinically effective ethanol-solution-gel, 2) the gel with propylene glycol and 3) the gel with RDP in the *ex vivo* porcine ear penetration study. Even though propylene glycol improved saturation solubility of nanocrystals, it could not bring benefit to nanocrystals in the penetration study. Based on these optimized azithromycin nanocrystals, topical administration for enhanced dermal bioavailability of azithromycin seems to be feasible.

1. Introduction

The rate of Lyme disease has been increasing in recent years around the whole world (Huber et al. 2010). People suffer from Lyme disease after infected-tick bite due to the bacterium *Borrelia burgdorferi* (Hinckley et al. 2014; Zimering et al. 2014). Serious symptoms on skin, joints and nervous system such as erythema migrans rashes, oligoarticular arthritis, and meningitis could happen when the Lyme disease was not treated promptly (Hinckley et al. 2014). As there is currently no vaccination available for Lyme disease (Piesman et al. 2014), therapeutic strategies focus on antibiotics. Usually, oral antibiotics such as doxycycline or amoxicillin are administered in the early treatment to patients who have been bitten by ticks (Shapiro 2014). However, bacterial resistance to administered antibiotic, malaise or toxicity has to be considered when using antibiotics systematically (Knauer et al. 2011). These side effects can be reduced when drug concentrations are kept at a minimum, and the antibiotic is administered topically.

Topical treatments with antibiotics such as amoxicillin, doxycycline and erythromycin against Lyme disease were studied in a rodent model (Shih and Spielman 1993). Erythromycin was the one working worst among those mentioned above. However, its derivative azithromycin (AZ) showed similar effectiveness in the treatment of early Lyme disease as amoxicillin but with less side effects (Massarotti et al. 1992). Azithromycin even possessed superior efficacy compared to doxycycline and erythromycin (Piesman et al. 2014). These advantages of azithromycin should be ascribed to its lower minimum inhibitory concentration and quicker onset time (Knauer et al. 2011; Piesman et al. 2014). Considering the difficult diagnosis in the early stage of Lyme disease and serious clinical signs in its late stage (Knauer et al. 2011; Piesman et al. 2014), quick initiation of a dermal azithromycin treatment after tick exposure should pave the way for preventing Lyme disease.

There are already optimized azithromycin topical formulation such as ethanol-solution-gel (Knauer et al. 2011; Schwameis et al. 2017) and azithromycin Lipoderm cream (Piesman et al. 2014). The former one was demonstrated effective in human clinical phase III trial and the latter one was effective in a murine model. But both of them

used azithromycin raw drug powder in their dermal vehicles, which would reduce dermal bioavailability due to the hydrophobicity of azithromycin (Mishra et al. 2009). Nanocrystals are assumed to be a good alternative herein. Their advantages on dermal application are ascribed to the nano size compared to micrometer sized raw drug powders: 1) The decreased particle radius brings enhanced saturation solubility due to Ostwald-Freundlich equation (Mauludin et al. 2009); 2) The smaller size results in greater curvature of the particle surface which brings in subsequent increased dissolution pressure and dissolution velocity according to Kelvin equation (Pardeike et al. 2010); 3) The nano size leads to enlarged surface area, and the increased saturation solubility leads to increased concentration gradient. In the end, they will facilitate the enhancement of dissolution velocity based on Noyes-Whitney equation (Müller et al. 2011). Both of the increased saturation solubility and increased dissolution velocity will lead to enhanced diffusive flux of the drug through the stratum corneum because of Fick's first law of diffusion (Zhai et al. 2014). Thus, improved dermal bioavailability is achieved due to the enhanced penetration (Müller et al. 2011). Moreover, nanocrystals can obtain increased adhesiveness to skin due to the enlarged contact area (Pyo 2016). Thus, this study was designed to optimize azithromycin nanocrystals for topical administration as well as to investigate the effect of nanonization on its anti-Lyme disease potential compared to the clinically effective ethanol-solution-gel and the gel with penetration enhancer.

2. Investigations, results and discussion

2.1. Optimized formulation of azithromycin nanocrystals

2.1.1. Screening of different surfactants and production parameters for the optimal size of azithromycin nanocrystals produced by HPH

Four nonionic surfactants (TPGS, poloxamer 407, Plantacare[®] 810 and Plantacare[®] 2000) were used to produce azithromycin nanocrystals. Nonionic surfactants are selected because of their lower

toxicity (Gibson 2009) and lower irritation potential compared to ionic surfactants (Ghosh et al. 2012). Considering the final particle size and the efficiency, 15 cycles at 1,500 bar was set as optimal parameter to produce 10.0 % azithromycin nanosuspension stabilized with 1.0 % TPGS, leading to a mean particle size of 427 nm with PDI of 0.403 (Fig. 1). In general, more homogenization cycles can lead to smaller particle sizes (Möschwitzer 2013). However, too much energy (more cycles) can promote aggregation during storage. The aggregation happened due to the acceleration of crystals resulting from the enhanced kinetic energy of the crystals (Kakran 2012). In Fig. 1, TPGS stabilized nanoparticles showed a decrease in PCS diameter until 15 cycles. Further cycles even resulted in slight increase of the particle size. Similar phenomenon that size increases by more homogenization cycles also happened in the case of Plantacare® 810 UP and Plantacare® 2000 UP.

2.1.2. Screening of different surfactants and production parameters for the optimal size of azithromycin nanocrystals produced by BM

By only 10 min wet bead milling of TPGS stabilized formulation PCS diameter of 189 nm and PDI of 0.194 were achieved (Fig. 2).

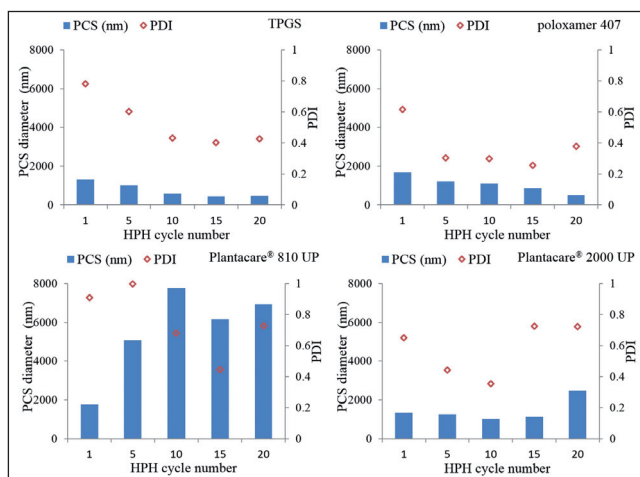


Fig. 1: PCS mean particle size and PDI of freshly produced azithromycin nanocrystals stabilized with TPGS, poloxamer 407, Plantacare® 810 UP or Plantacare® 2000 UP, in dependence of applied HPH cycle number.

Increasing the milling time led to gelation of the formulation. Also with poloxamer 407 azithromycin nanocrystals with PCS diameter of 185 nm and PDI of 0.234 could be achieved after a slightly longer milling time of 15 min. The lower PDI of the TPGS stabilized formulation compared to the poloxamer 407 formulation represents a narrower size distribution and thus often a higher physical storage stability due to slower Ostwald ripening (Müller and Jacobs 2002). The nanosuspension stabilized with Plantacare® 810 UP and Plantacare® 2000 UP gelled after 5 min milling. The reason could be agglomeration or crystal growth of very small particles resulting from the thermodynamically unstable nanosuspension (supersaturation). This phenomenon occurred with the change of the Gibbs free energy of the system during the milling process (Ghosh et al. 2012). Furthermore, in the cases of poloxamer 407 and Plantacare® 2000 UP, enhancement of the particle size occurred with increased milling time analogous to HPH method. This was due to the additional input of energy enhancing the kinetic energy of the crystals causing aggregation (Kakran et al. 2012).

2.1.3. Production of nanocrystals by combination technology (smartCrystals-technology)

The best two formulations produced by BM – one stabilized with TPGS (milling time 10 min) and another with poloxamer 407

(milling time 15 min) – were further processed by 1 HPH cycle at 300 bar and 5 °C. The particle sizes and zeta potentials of the obtained nanocrystals produced by the combination technology are

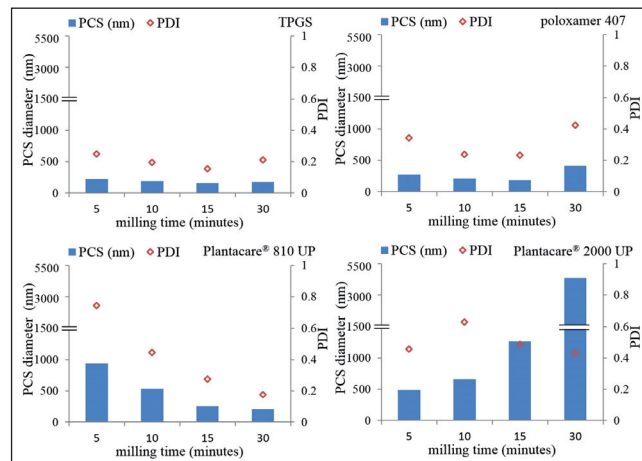


Fig. 2: PCS mean particle size and PDI of freshly produced azithromycin nanocrystals stabilized with TPGS, poloxamer 407, Plantacare® 810 UP or Plantacare® 2000 UP, in dependence of bead milling time (min).

shown in Fig. 3, as well as the corresponding data of BM products. In both TPGS and poloxamer 407 stabilized nanosuspensions, subsequent processing by HPH in the combination technology did not make a significant difference ($P < 0.05$) either in particle size (PCS, $d(v)50\%$, $d(v)90\%$) nor in particle size distribution (PDI). In considering the extra energy cost of the subsequent HPH production in the combination technology, the suggested method to produce azithromycin nanocrystals in this study is bead milling. TPGS and poloxamer 407 would still provide steric stabilization (Pardeike and Müller 2010) for the azithromycin nanosuspension.

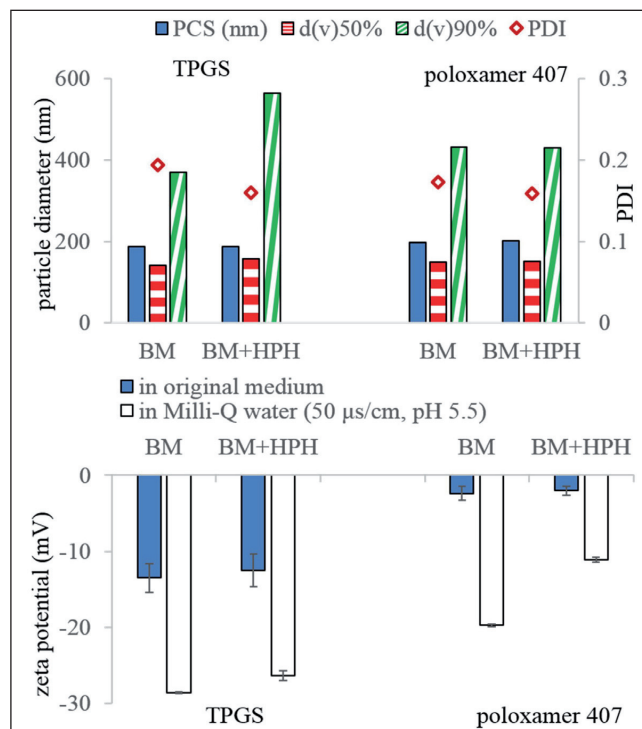


Fig. 3: Particle size (PCS diameter, LD diameter $d(v)50\%$, $d(v)90\%$) and PDI (upper) as well as zeta potential (lower) measured in original medium and in conductivity adjusted Milli-Q water of azithromycin nanocrystals stabilized by either TPGS or poloxamer 407 and produced by 2 methods on day 0.

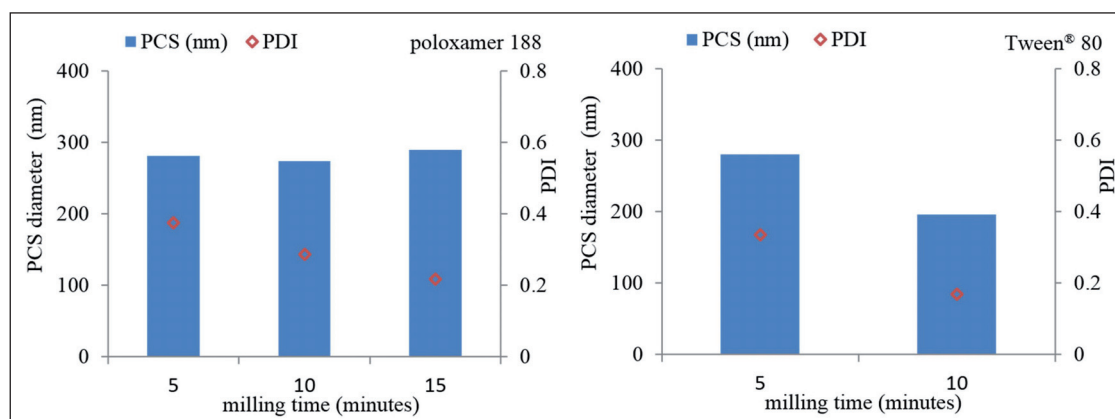


Fig. 4: PCS mean particle size and PDI of freshly produced azithromycin nanocrystals stabilized with poloxamer 188 or Tween® 80 in dependence of bead milling time (min).

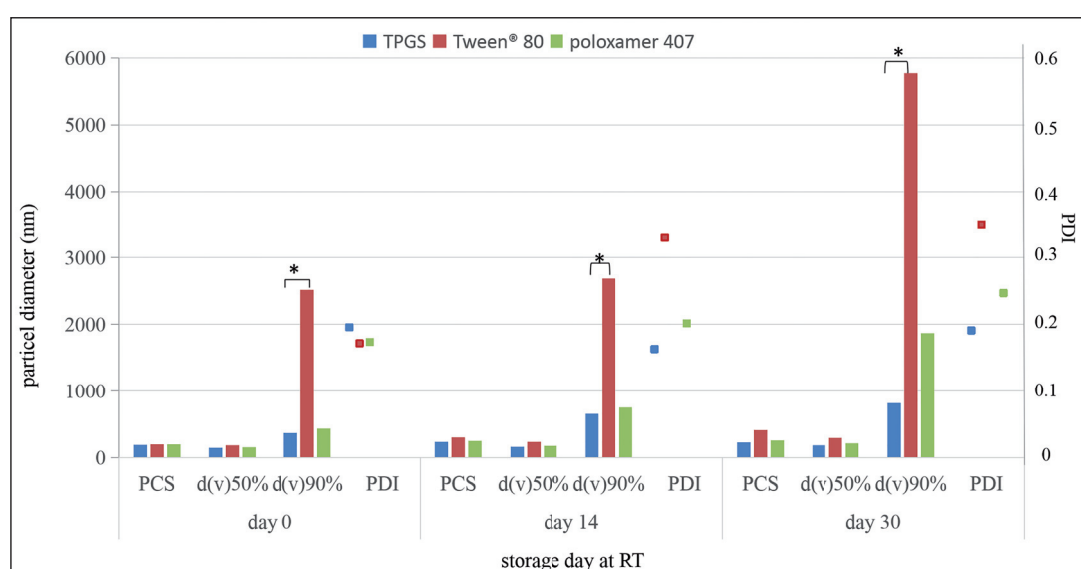


Fig. 5: Particle size (PCS diameter, LD diameter d(v)50%, d(v)90%) and PDI of azithromycin nanocrystals stabilized with TPGS, Tween® 80 or poloxamer 407 over a period of 30 days at RT (*P<0.05).

sion even though the measured zeta potential were less than -30 mV (Fig. 3) which is usually considered as a level to predict the physical storage stability (Al-Shaal 2011). And the stability was confirmed in section 2.1.5.

2.1.4. Further surfactants screening for azithromycin nanocrystals produced by BM

Two popular nonionic surfactants poloxamer 188 and Tween® 80 were further used to stabilize azithromycin nanocrystals produced by the best method – bead milling. There was no significant difference ($P>0.05$) between the product stabilized by Tween® 80 and by poloxamer 188. But it only cost 10 min to obtain nanocrystals with PCS particle size smaller than 200 nm and PDI smaller than 0.2 when the stabilizer is Tween® 80 (Fig. 4). While for poloxamer 188, no particle size smaller than 200 nm was detected even after 15 min production. Thus, to save production time, Tween® 80 was selected for further investigations together with TPGS and poloxamer 407.

2.1.5. Short-term stability of nanocrystals stabilized with TPGS, poloxamer 407 and Tween® 80

Azithromycin nanocrystals stabilized with TPGS, poloxamer 407 or Tween® 80 were produced by bead milling for 10, 15 and 10 min, respectively. Even though Tween® 80 stabilized azithromycin nanocrystals had almost similar PCS mean diameter compared with the

TPGS stabilized formulation (Fig. 5), large particles or aggregates ($d(v)90\% > 2\ \mu\text{m}$) were detected by LD already on day 0. Why the PCS mean particle size is almost similar for both formulations even though the Tween® 80 stabilized formulation had particles larger than $2\ \mu\text{m}$ can be explained by the different detecting ranges of both methods. PCS is not prone to detect the presence of large particles or aggregates as good as LD is (Romero et al. 2016). In contrast, the formulation stabilized with TPGS had no pronounced alterations for both PCS and LD values over a period of 30 days at RT being still in nanometer dimension (PCS diameter < 240 nm with PDI < 0.2 and $d(v)90\% < 1\ \mu\text{m}$). Considering the significant difference between the $d(v)90\%$ of production stabilized by TPGS and the one by Tween® 80 ($P<0.05$), it is suggested that TPGS is the optimal surfactant to stabilize azithromycin nanocrystals. Therefore, the physically most stable azithromycin nanocrystals at 10.0 % (w/w) can be obtained by TPGS stabilization at 1.0 % (w/w) and 10 min bead milling at $5\ ^\circ\text{C}$, and was selected as the best formulation in this study.

2.2. Characterization of the optimal formulation

2.2.1. Transmission electron microscope (TEM)

The morphological characterization of the optimal formulation after 30 days storage at room temperature (RT) is shown in Fig. 6. It is obvious to clarify that most of the azithromycin nanocrystals produced by BM were in shape considering the black spots

captured by the TEM. The size given by TEM (circled in red) was spherical consistent with the $d(v)90\%$ value shown in Fig. 5. The observation convinced that no big particles beyond the nano range of the optimal nanocrystals appeared after 30 days storage at RT.

2.2.2. X-ray diffraction (XRD)

X-ray diffraction patterns of the raw drug powder (RDP) and optimal azithromycin nanocrystals (NC) can be found in Fig. 7. From RDP analysis by X-ray scattering, two main characteristic crystalline peaks of azithromycin were found at 9.96° and 18.8°

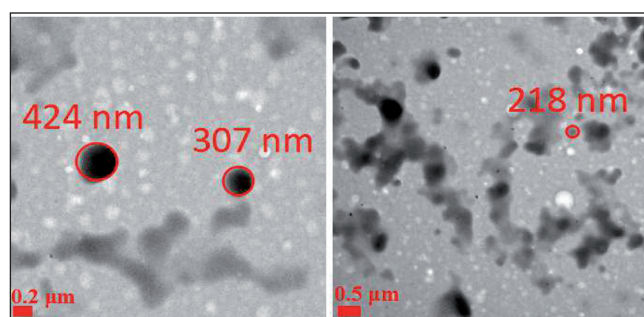


Fig. 6: TEM micrographs of the optimal azithromycin nanosuspension after 30 days storage at RT (scale bar left: 0.2 μm , and right: 0.5 μm).

(Fig. 7, blue graph). The NC also had peaks at both positions, indicating that bead milling did not affect the crystal state of azithromycin. The increase of the baseline of nanocrystals from 20 degree (deg) to 40 degree is due to the water inside of the nanosuspension.

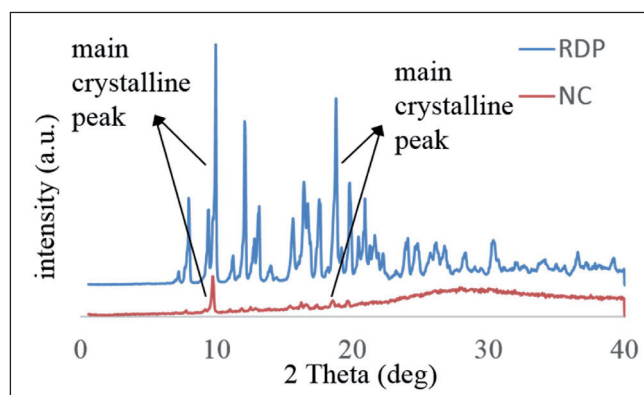


Fig. 7: X-ray diffraction patterns of azithromycin RDP (blue) and optimized azithromycin nanosuspension (NC, red).

2.3. Optimal storage temperature of nanocrystals stabilized with TPGS

Physical short-term stability data of TPGS stabilized azithromycin nanocrystals produced by 3 different methods and stored at 3 different temperatures (fridge ($4 \pm 2^\circ\text{C}$), room temperature (RT, $20 \pm 2^\circ\text{C}$), oven ($40 \pm 2^\circ\text{C}$)) are shown in Fig. 8. The samples produced by BM showed high physical stability at all temperatures. Only for the azithromycin nanocrystals produced by BM, the particle size stayed still in nanometer range (PCS diameter < 300 nm with $\text{PDI} < 0.25$ and $d(v)90\% < 1 \mu\text{m}$) over a storage time of 90 days independently from the storage temperature. The sample stored at 40°C even showed the smallest PCS diameter of 215 nm and $d(v)90\%$ of 0.872 μm after 90 days. The reduced particle size of the production in high temperature can be explained by the fact that solubility of azithromycin nanocrystals increased with increased temperature (Mishra et al. 2009). However, big particles

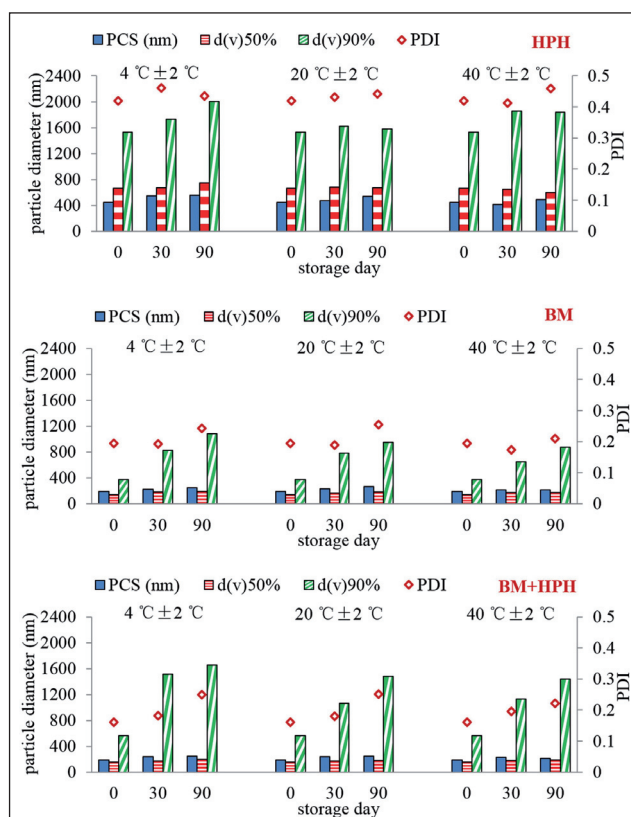


Fig. 8: Particle size (PCS diameter, LD diameter $d(v)50\%$, $d(v)90\%$) and PDI of TPGS stabilized azithromycin nanocrystals produced by 3 methods over a period of 90 days at 4°C , RT and 40°C .

will appear when temperature cools down for use. Because the small particles in the suspension will dissolve faster, re-crystallization happen on the surface of the bigger particles. Moreover, only the nanosuspension stabilized with TPGS and stored at 40°C changed its color from white to yellow at all storage temperatures (Fig. 9 upper) or in all the productions with other stabilizers stored at 40°C (Fig. 9 middle). The explanation could be the pH (8.5) of the 1.0 % TPGS stabilized azithromycin nanosuspension resulting in the hydrolysis of the ester bond of TPGS during the storage time of 90 days at 40°C , leading to a more yellow tocopheryl quinone (Brigelius-Flohé and Traber 1999). This explanation was confirmed by the colour change of 1% TPGS solution during storage at 40°C . All in all, 40°C is not a suggested temperature to store azithromycin nanosuspensions. For the other two temperatures, the sample stored at RT showed $d(v)90\%$ of 2.305 μm only after 6 months storage, while that stored at 4°C only showed $d(v)90\%$ of 1.250 μm over a storage time of 24 months (Fig. 10 A). In addition, the samples have been subjected to microbial attack when stored at RT after 12 months (Fig. 9 lower). In the opposite, samples avoided microbial attack when stored in the fridge for up to 36 months. Thus, $4 \pm 2^\circ\text{C}$ is the suggested storage condition for TPGS stabilized azithromycin nanocrystals in considering of both particle size and antimicrobial efficacy.

2.4. Physical long-term stability of the optimal formulation

The physical long-term stability of the optimized formulation up to 36 months at 4°C was analyzed by PCS, LD (Fig. 10 A) and under a light microscope (Fig. 10 B). Sediment appeared after 12 months, but it was easily re-dispersible by shaking for 10 s by hand. The longer the storage time, the more shaking was necessary for re-dispersion. During the storage, the $d(v)90\%$ of the optimal nanocrystals was detected far beyond the nanorange ($>>1000 \text{ nm}$) after 36 months. This phenomenon could be explained by the aggregation of big particles. These data detected by PCS and LD are consistent with light microscope pictures shown in Fig.

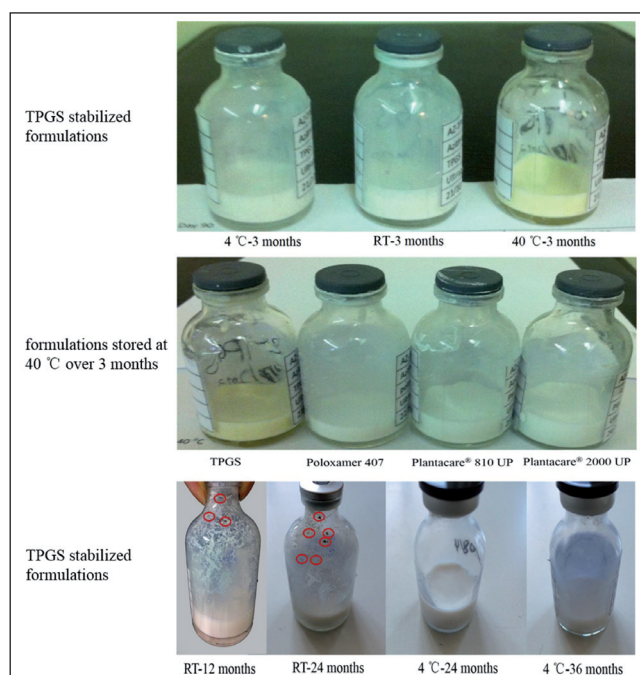


Fig. 9: Macroscopic appearance of azithromycin nanosuspension stabilized with TPGS and stored for 3 months at 3 different temperatures (upper); azithromycin nanosuspension stabilized with 4 different surfactants and stored at 40 °C for 3 months (middle) and TPGS stabilized azithromycin nanosuspension stored at either 4 °C or RT over a period up to 36 months (lower) with visible microbial growth, framed red.

10 B. Light microscope does possess the ability to monitor crystal growth (Steele 2009). Homogeneous dispersed nanoparticles were observed by light microscope for the best formulation up to 24 months.

2.5. Dissolution test and saturation solubility

The best nanocrystals (NC) and RDP were dispersed in Milli-Q water for the dissolution test. RDP in water with additional TPGS at identical concentrations used for stabilizing the nanosuspension

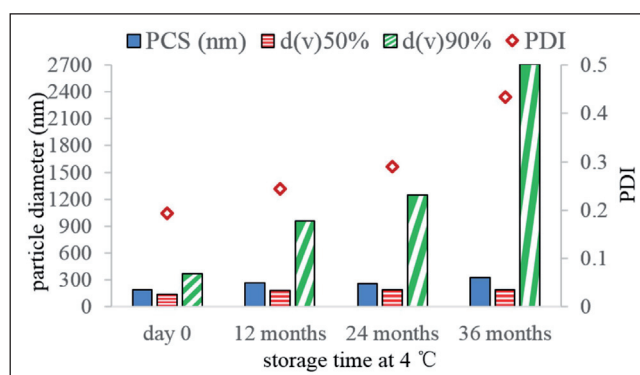


Fig. 10: (A) Particle size (PCS diameter, LD diameter $d(v)50\%$, $d(v)90\%$) and PDI of TPGS stabilized azithromycin nanocrystals produced by BM and stored at 4 °C over a period of 36 months.

was also tested to exclude the influence of TPGS. According to Fig. 11 A, the addition of TPGS in water increased the dissolved amount of an azithromycin RDP slightly, whereas NC had around 2-3 times more dissolved azithromycin than RDP at all measuring points during the 8 h investigation period. Obviously, the dissolution velocity of NC was higher than RDP (200 $\mu\text{g}/\text{ml}$ for NC and 71 $\mu\text{g}/\text{ml}$ for RDP after 1 h), which can be mainly ascribed to

Pharmazie 74 (2019)

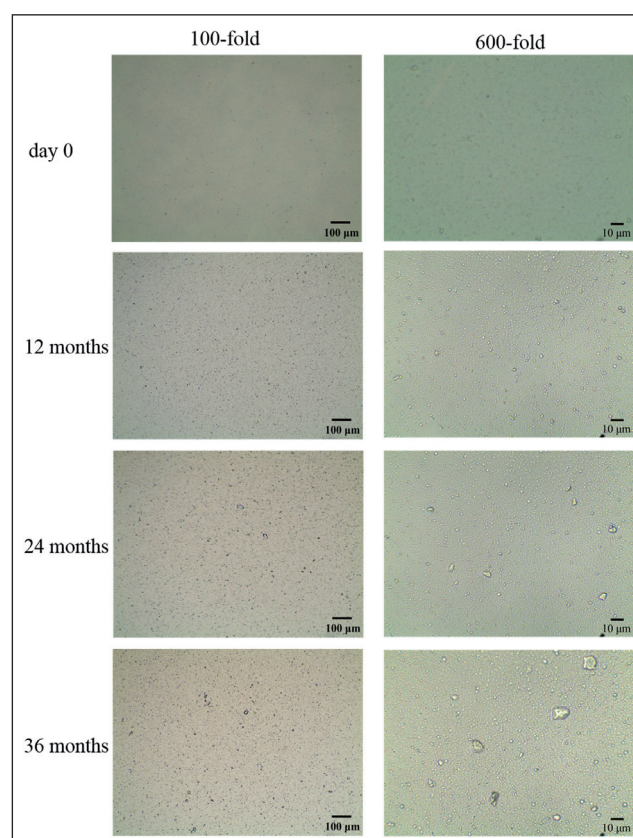


Fig. 10: (B) Light microscope pictures at 100-fold magnification (left) and at 600-fold magnification (right) of TPGS stabilized azithromycin nanocrystals produced by BM after production (day 0), 12 months, 24 months and 36 months storage at 4 °C (scale bars from left to right: 100 μm and 10 μm).

the nano size following the Noyes Whitney equation (Müller et al. 2013). It is well known that the nanometer-sized particles have an increased saturation solubility compared to its micrometer-sized counterpart according to Ostwald-Freundlich equation and Kelvin equation (Mauludin et al. 2009; Pardeike et al. 2010). This can be seen from Fig. 11 A, where the amount of dissolved azithromycin is 3 times higher in the plateau area (242 $\mu\text{g}/\text{ml}$ of NC versus 85 $\mu\text{g}/\text{ml}$ of RDP at 4 h).

Propylene glycol (PG) is one of the most commonly used penetration enhancer/co-solvent to help drugs dissolve more and penetrate better (Moser et al. 2001). It is approved for dermal use due to its non-toxicity and well-tolerance (Mujica Ascencio et al. 2016; Herkenne et al. 2008). PG is also one of the humectants, which can increase the moisturizing and occlusive effect of gels (Brown et al. 2012). Moreover, PG is a low dielectric which can prevent crystal growth (Zhai 2013). PG (20 % w/w) was added into the original dispersion medium Milli-Q water to test the influence of PG on saturation solubility of azithromycin. The concentration (20 % w/w) was selected because the addition of 20 % PG would not change the viscosity of the original gel too much (data not shown here). In addition, 20 % PG could act as a preservative (Prickett et al. 1961). More details of the influence of the percentage of the PG on drug can be found elsewhere (Herkenne et al. 2008; Moser et al. 2001).

It can be concluded from the results displayed in Fig. 11 B that, in general, azithromycin dissolves much better in the dispersion medium containing PG than in pure water. The saturation solubility of the NC in the water-PG mixture (NC in PG (20%)) was 2,648 $\mu\text{g}/\text{ml}$ at 4 h, and that of the RDP in the water-PG mixture (RDP in PG (20%)) was 2,403 $\mu\text{g}/\text{ml}$ at 8 h. The addition of TPGS did not influence in this dispersion medium. The data was reasonable because PG is known to influence the solubility of drugs in a vehicle (Mujica Ascencio et al. 2016; Smith et al. 2001). The influence of PG in the dermal gel was further investigated as following.

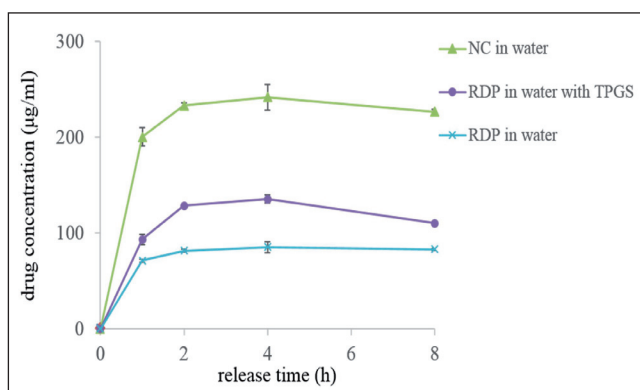


Fig. 11: (A) Dissolution profiles of the azithromycin nanocrystals (NC) in water, compared with RDP in the same dispersion medium at 25 °C after shaking of 8 h (n=3). RDP in water with TPGS was also tested herein for assessing the influence of TPGS in water.

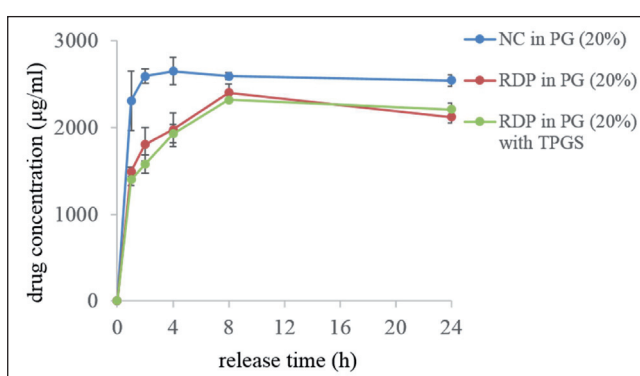


Fig. 11: (B) Dissolution profiles of the azithromycin nanocrystals (NC) in water-PG mixture (80:20, w/w) (in short: PG (20%)), compared with RDP in the same dispersion medium at 25 °C after shaking of 24 h (n=3). RDP in PG (20%) with TPGS was also tested herein for assessing the influence of TPGS in the dispersion medium.

2.6. Characterization of nanocrystals incorporated gels

The five gels were characterized by light microscope. Gel A (10.0 % AZ-ethanol-solution gel) contained RDP and ethanol which showed efficacy to treat tick infection (Knauer et al. 2011). Gel B (5.0 % AZ-nano-HPC gel) was composed of 5.0 % azithromycin nanocrystals with a PCS diameter of 189 nm in the 5.0 % HPC gel. Gel C (5.0 % AZ-nano-PG-HPC gel) contained extra 20.0 % PG compared to gel B. RDP was replaced by nanocrystals to get Gel D (RDP gel without PG) and Gel E (extra 20.0 % PG). The observations over a period of 14 days done by light microscope (Fig. 12) are as follows: azithromycin should be completely dissolved in gel A due to the high ethanol content of 77.5% (w/w), confirmed by the microscopic pictures at day 0. However, crystal growth could clearly be observed at day 7 and day 14. The explanation could be that azithromycin dissolved totally in gel A with the 77.5% (w/w) ethanol in the beginning. But with ethanol evaporated in the air naturally, the state of the azithromycin in the gel A changed from dissolved state to supersaturation which is thermodynamically unstable and resulted in re-crystallization (Moser et al. 2001; Steele 2009). This phenomenon was also observed by McHugh et al. (2004), who concluded that an ethanol/water mixture with 100%, 90%, 80% and 70% (w/w) ethanol will cause crystallization or precipitation. Thus, adding volatile ethanol in the gel (gel A) may be helpful to dissolve the hydrophobic active in beginning, but it is not a suggested method because re-crystallization may happen along with the natural evaporation of ethanol.

In consideration of the high saturation solubility of azithromycin RDP/NC in PG (20 %) (Fig. 11 B), 20 % PG added into the gel was expected to keep more dissolved drug in the gel base. However, big particles were visible in this case of gel C (nanocrystal inside

the gel mixed with 20 % PG) at day 7 and day 14 because the supersaturated system is prone to accelerate crystal growth (Moser et al. 2001; Steele 2009). In addition, gel E (RDP inside the gel mixed with 20 % PG) turned out possessing many big crystals in day 0. Their particle size decreased with storage time due to their solubility in PG. In contrast, not a big difference was observed for gels without PG during the storage time: gel B (nanocrystal incorporated in the gel without PG) and gel D (RDP incorporated in the gel without PG). Thus, addition of the penetration enhancer PG is not a good way to keep the particle size of azithromycin nanocrystals stable in the HPC gel base.

2.7. Ex vivo porcine ear penetration study

The total amount (µg) of azithromycin removed with each tape is summarized in Fig. 13. The first tape was excluded from the analysis since almost the non-penetrated formulation on the skin

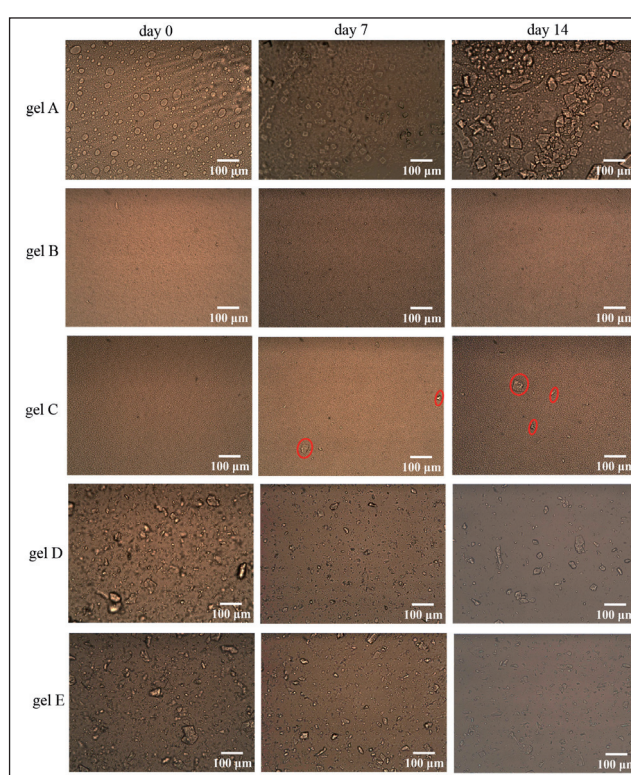


Fig. 12: Light microscope pictures at 160-fold magnification of gel A (ethanol-solution-gel), gel B (best nanocrystal in gel without PG), gel C (best nanocrystal in gel with PG), gel D (RDP gel without PG) and gel E (RDP gel with PG) directly after production (day 0), and after 7 and 14 days storage at room temperature (scale bar: 100 µm).

surface was removed with it. Gel C (best nanocrystal in gel with PG) was expected to have higher penetration efficacy than gel B (best nanocrystal in gel without PG), regarding its higher saturation solubility of azithromycin shown in Fig. 11 and additionally due to the penetration enhancing property of PG (Smith 2001). Surprisingly, the result was the opposite: azithromycin from gel B penetrated deeper with higher active concentrations compared to gel C according to Fig. 13. There are two possible explanations. One explanation is that the supersaturated situation of nanocrystals in gel with PG facilitated crystal growth in gel C (Steele 2009). This assumption could be confirmed by the observations presented in Fig. 12. Another explanation is that PG penetrated faster than the drug after being applied on the skin. This assumption was derived from the observation by confocal Raman microscope (Mujica Ascencio et al. 2016) and by stimulated Raman scattering microscope (Saar et al. 2011). In that case, PG would penetrate faster and leave nanocrystals behind in the upper part of the skin.

With the loss of PG as solvent the azithromycin precipitated and penetration was further weakened. However, only the second explanation can additionally explain the phenomenon that gel C (best nanocrystal in gel with PG) penetrated even less than gel E (RDP in gel with PG). Because nanocrystals had higher solubility than RDP in the mixture of water and PG mentioned in Fig. 11, the absence of PG resulted in more precipitation of nanocrystals followed by less penetration.

Gel A, the one which was demonstrated to be clinically effective (Knauer et al. 2011; Schwameis et al. 2017) only showed the higher penetration amount than gel B in tape 2 and tape 3 (Fig. 13). However, it should be kept in mind that gel A was the one with 10.0% (w/w) active while each of the other gels contained only 5.0% (w/w). In considering the penetration amount in other single deeper tape (tape 4-30) (Fig. 13), gel B (best nanocrystal in gel without PG) was doubtlessly the best performing one in the penetration study among the 5 gels. This benefited from the stable high solubility provided by the nanocrystal in the gel and increased concentration gradient between the gel and the skin (Zhai et al. 2014). At the end, penetration ability was increased.

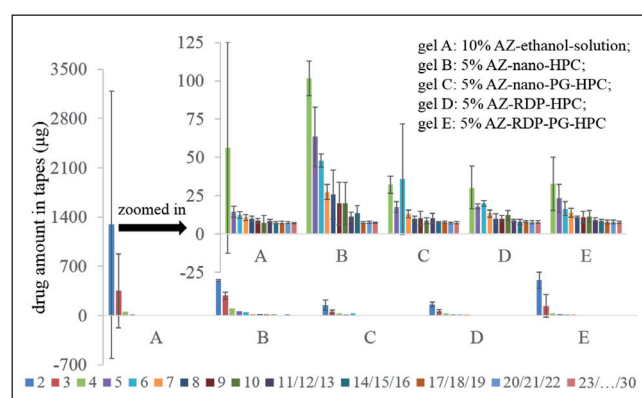


Fig. 13: Total amount of azithromycin in tapes after 20 minutes penetration (n=3). Gel A: ethanol-solution-gel; gel B: best nanocrystal in gel without PG; gel C: best nanocrystal in gel with PG; gel D: RDP gel without PG; gel E: RDP gel with PG.

3. Experimental

3.1. Materials

Azithromycin (AZ) in short for azithromycin dihydrate (contains 95.4% azithromycin) was bought from Haohua Industry (Jinan, China). Capryl glucoside (Plantacare® 810 UP), decyl glucoside (Plantacare® 2000 UP), poloxamer 188 (Kolliphor® P188), poloxamer 407 (Kolliphor® P407) and D- α -tocopheryl polyethylenglycol 1000 succinate (Kolliphor® TPGS) were kindly provided from BASF SE (Ludwigshafen am Rhein, Germany). Hydroxypropyl cellulose (Klucel GF®), viscosity of 2% in water at 25 °C 300 mPa s, MW 370,000 Da), Miglyol® 812, and polysorbate 80 (Tween® 80) were purchased from Caesar & Loretz GmbH (Hilden, Germany) and 1,2-Propanediol (propylene glycol) by Alfa Aesar GmbH & Co. KG (Karlsruhe, Germany). Yttrium-stabilized zirconium oxide milling beads having a diameter of 0.1 mm were obtained from Hosokawa Alpine (Augsburg, Germany). Ethanol at 96% was used of analytical grade. The Milli-Q water was obtained by reverse osmosis from a Millipak® Express 20 Filter unit (MPGP02001, Merck, Millipore KGaA, Darmstadt, Germany).

3.2. Optimized formulation of azithromycin nanocrystals

3.2.1. Optimized production by high pressure homogenization (HPH)

The high pressure homogenizer Micron LAB 40 (HPH, APV GmbH, Mainz, Germany), equipped with a water jacket for temperature control, was first used to produce azithromycin nanocrystals. The formulation contained 10.0% (w/w) azithromycin and 1.0% (w/w) surfactant dispersed in Milli-Q water. Pre-suspension was produced by suspending the coarse azithromycin powder in the aqueous surfactant solution via high shear mixing using an Ultra-Turrax T25 (Janke & Kunde GmbH & Co. KG, Germany) for 1 min at 8,000 rpm. The so obtained pre-suspension was first homogenized by HPH at low and moderate pressures (250 and 500 bar, 2 cycles each) and subsequently at 1,500 bar for 20 homogenization cycles. The whole homogenization process was performed at 5 °C. Particle size was monitored in dependence of homogenization cycles after 1, 5, 10, 15 and 20 cycles.

3.2.2. Optimized production by bead milling (BM)

Bead milling (BM) was also applied to produce azithromycin nanosuspensions to compare the results with the HPH method. Pre-suspensions with the same composition were identically produced as in section 3.2.1. Next, wet bead milling was performed with yttrium-stabilized zirconium oxide beads (0.1 mm diameter) using a discontinuous propeller mill (PML 2, Bühler AG, Uzwil, Switzerland) at a rotation speed of 2,000 rpm. The process was performed at 5 °C by controlled circulation of cooled water through an outer jacket. Particle size was analyzed in dependence of milling time after 5, 10, 15 and 30 min.

3.2.3. Production of nanocrystals by combination technology (CT)

Combination technology (smartCrystals-technology) (Pyo 2016) with the combination of bead milling and HPH was performed in this study to produce nanocrystals as well. The best two receipts selected from the bead milling method were first possessed by bead milling, a relatively low energy process (Al-Shaal et al. 2011) as described in section 3.2.2. In the second subsequent step, these nanosuspensions were passed through HPH (relatively high energy process) at low pressure of 300 bar for 1 cycle at 5 °C to further reduce the particle size for increased physical storage stability.

3.3. Preparation of dermal azithromycin formulation

3.3.1. Preparation of azithromycin ethanol-solution-gel as reference

Ethanol-solution-gel consisted of 10.0% azithromycin raw drug powder (RDP), 77.5% ethanol (94%), 0.5% polyacrylate, 5.0% HPC and 7.0% Miglyol® 812 was produced as described (Knauer et al. 2011). This formulation was selected as reference (gel A) due to its demonstrated effectiveness in clinical studies up to phase III (Schwameis et al. 2017).

3.3.2. Preparation of azithromycin nanocrystals containing dermal gel

Hydroxypropyl cellulose (HPC) powder at 10.0% (w/w) was added to 75 °C hot Milli-Q water respectively in a mortar, followed by stirring using a pestle to obtain a fine suspension (10.0% HPC gel base). After 24 h in a fridge (4±2 °C), a transparent gel base free of lumps and air bubbles was obtained. Evaporated Milli-Q water during production and storage was added as last step to the gel base. The best azithromycin nanosuspension at 10.0% (w/w) was incorporated into the 10.0% HPC gel base with a final concentration of 5.0% azithromycin and 5.0% (w/w) HPC (gel B). To obtain gel C and gel E, propylene glycol (PG), a common penetration enhancer (Watkinson et al. 2009), was first mixed with the gel base to get a homogeneous appearance, followed by the mixture with the best formulation (gel C) or RDP (gel E) to obtain a 5.0% (w/w) HPC gel contains 20.0% (w/w) PG and 5.0% (w/w) azithromycin. The gel with RDP but without penetration enhancer (gel D) was also prepared as comparison. Surfactant was added in the gel to exclude its influence when compared the difference between nanosuspension and RDP. The final amount of each excipient for gel B – gel D is listed in the Table.

PG: propylene glycol; HPC: hydroxypropyl cellulose; TPGS: D- α -tocopheryl polyethylenglycol 1000 succinate; RDP: raw drug powder

Table: Composition (w/w) of azithromycin HPC gels

Gel	Azithromycin 5.0% (w/w)	PG (%w/w)	HPC (%w/w)	TPGS (%w/w)	Purified water (%w/w)
B	nanocrystal	-	5.0	0.5	89.5
C	nanocrystal	20.0	5.0	0.5	69.5
D	RDP	-	5.0	0.5	89.5
E	RDP	20.0	5.0	0.5	69.5

3.4. Characterization

3.4.1. Photon correlation spectroscopy (PCS)

Photon correlation spectroscopy (PCS, Zetasizer Nano ZS, Malvern Instrument, UK) was used to measure the mean particle diameter and polydispersity index (PDI) of nanocrystals by dynamic light scattering. The PCS diameter stands for the intensity weighted mean diameter of the bulk population and the PDI represents the width of the size distribution. For the measurement 10 μ l nanosuspension was diluted in 10 ml Milli-Q water. 10 single measurements were done for one sample and the mean calculation. The detectable size range of PCS is from approximately 3 nm to 3 μ m (Romero et al. 2016).

3.4.2. Laser diffractometry (LD)

Laser diffractometry (LD, Mastersizer 2000, Malvern Instrument, UK) was used to detect large particles or aggregates beyond the PCS measuring range by static light scattering. Its measuring range is from around 20 nm to 2,000 μ m (Romero et al. 2016). The results were displayed by volume weighted diameters d(v)50% and d(v)90%, meaning that 50% or 90% of the particles were below the given value of d(v)50% or d(v)90%, respectively. The real refractive index of 1.532 and imaginary refractive index of 0.01 were used for azithromycin and real refractive index of 1.330 was used for water to calculate the volume diameters following the Mie theory.

3.4.3. Zeta potential (ZP)

Zeta potential (ZP) represents the electrostatic charge on the surface of the particle (Pyo et al. 2016) and can be converted from the electrophoretic mobility of a particle (Müller and Jacob 2002), determined by electrophoretic light scattering in the Zetasizer Nano ZS (Malvern Instrument, UK). It can be used to predict the physical stability of colloidal suspensions, where absolute values higher than 30 mV represent an electrostatically good stabilized system. The measurements were performed in two different media: conductivity adjusted Milli-Q water (50 µS/cm, pH 5.5), and original medium (aqueous phase of the formulation).

3.4.4. Light microscope (LM)

A light microscope (BA210LED) equipped with a digital camera (Moticam 3.0 MP, both from Motic Deutschland GmbH, Germany) was used at magnifications of 100 and 600 to detect large particles or aggregates within nanosuspensions. Another light microscope (Orthoplan, Leitz, Germany) equipped with a digital camera (CMEX-1, Euromex microscope, Netherlands) was used for the detection of any microcrystals in gel samples at a magnification of 160.

3.4.5. Transmission electron microscope (TEM)

The morphological examination of the optimal formulation was performed by a high resolution transmission electron microscope (TEM, Tecnai™ G² 20 S-TWIN, FEI Co., USA). Nanosuspension (5 µl) was dropped on copper grids (400 mesh) with hydrophilized carbon films and stained with 1% (w/v) uranyl acetate. The supernatant liquid was removed by filter paper and the left grid air-dried. An acceleration voltage of 160 kV was performed in the TEM analysis.

3.4.6. X-ray diffraction (XRD)

The crystalline state of the optimal formulation and RDP was estimated by X-ray diffraction (XRD, Philips PW 1830, Netherlands). The experiments were performed in symmetrical reflection mode with a Cu-Kα line as the source of radiation. Standard runs using a 40 kV voltage, a 25 mA current and a scanning rate of 0.04°/2 seconds over a range of 0.6–40° were applied. The tested nanosuspension was mixed with a guar gum (1.5% w/w) until creamy and put into a sample holder with a volume of 0.06 ml. The RDP was directly put into the sample holder.

3.5. Dissolution test and saturation solubility

Dissolution test and saturation solubility study were performed using an Innova 4230 shaker (New Brunswick Scientific GmbH, Nürtingen, Germany) at 25 °C and 100 rpm. Besides Milli-Q water, water-propylene glycol mixture (20% propylene glycol w/w) was also selected as dispersion medium. Samples were filled in 40 ml glass vials and the dispersion medium added, resulting in an azithromycin concentration of 2% (w/w). All vials were sealed with caps to avoid evaporation during shaking. 2 ml sample was withdrawn from each vial using a syringe after 1, 2, 4, 8 and 24 h and an equivalent amount of dispersion medium was replenished. All withdrawn samples were diluted immediately 2 times by the dispersion medium to avoid re-crystallization. Un-dissolved drug was separated by Amicon® ultra centrifugal filter units with a molecular weight cut-off of 3,000 Da (Millipore Ireland Ltd, Tullagreen, Ireland) at 2,082 × g (Sigma 3K18 centrifuge, Harz, Germany) at room temperature for 15 min. The azithromycin concentration of particle free solution passed through the filter representing the amount of dissolved drug was analyzed by HPLC. All experiments were done in triplicate.

3.6. Ex vivo porcine ear penetration study

The stratum corneum of porcine ear skin is demonstrated to be suitable for human dermatological research due to its layer thickness and morphological similarity (Gallagher 2005). Freshly excised porcine ears were obtained from domestic pigs in a local abattoir. The ears were washed and hairs removed by careful shaving before the performance of the penetration assay. 100 mg of each gel (gel A – gel E) was evenly applied onto the porcine ear skin having an area of 1.5 × 1.5 cm². After 20 min penetration time, adhesive tapes (tesafilm® crystal clear, No. 57315, 10 m × 15 mm, teas SE, Norderstedt, Germany) were pressed onto the treated areas using a roller for evenly distributed pressure. The skin was stripped sequentially and the removed tapes were collected in individual glass vials. 30 tapes in total were used to remove the stratum corneum on one with sample treated area. The azithromycin in each tape was extracted in 2 ml acetonitrile/DMSO (1:1, v/v) mixture by shaking (Innova 4230 shaker, New Brunswick Scientific GmbH, Nürtingen, Germany) for 3 h at 120 rpm and 25 °C. Afterwards, the extracts were centrifuged at 15,493 × g for 15 min (Heraeus Megafuge Centrifuge 3.0R, Kendro Laboratory Products GmbH, Hanau, Germany) and the so obtained supernatant was analyzed by HPLC.

3.7. High performance liquid chromatography (HPLC) analysis

Azithromycin concentration was determined by HPLC with an auto sampler model 560, equipped with a solvent delivery pump system model 522 and a diode array detector model 540 (Kontron Instruments, Rossdorf, Germany). As column a Lichrospher-100 RP-8 reverse phase column (125 × 4 mm, 5 µm) was used. As mobile phase, a buffer/methanol mixture at a ratio of 20:80 (v/v) was used with a flow rate of 1.2 ml/min, where the buffer was composed of 4.35 g/L K₂HPO₄ and H₃PO₄ of pH 7.5. The column temperature was set to 45 °C. The UV detector measured azithromycin at a wavelength of 210 nm.

Conflict of interest: None declared.

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