College of Pharmacy and Medical Technology[1], Putian University, Fujian, China; Institute of Pharmacy, Department of Pharmaceutics, Biopharmaceutics & NutriCosmetics[2], Freie Universität, Berlin; Institut für Pharmazeutische Technologie und Biopharmazie[3], Philipps University, Marburg; PharmaSol GmbH[4], Berlin, Germany

Maximum loaded amorphous azithromycin produced using the wetness impregnation method with fractional steps for dermal prophylaxis against Lyme disease

Nan Jin[1], Sung Min Pyo[2], C. M. Keck[3,4], R. H. Müller[2,*]

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**Corresponding author: Rainer H. Müller, Institute of Pharmacy, Department of Pharmaceutics, Biopharmaceutics & NutriCosmetics, Freie Universität Berlin, Kelchstr. 31, 12169 Berlin, Germany*
rainer.mueller@fu-berlin.de

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Azithromycin was loaded onto the µm-sized mesoporous silica Davisil® SP53D-11920 using the wetness impregnation method with fractional steps (WIFS) and further incorporated into a 5 % hydroxypropyl cellulose gel to prevent Lyme disease. Maximum loadings (32.0 % w/w and 33.2 % w/w) were produced by different concentrated loading solutions and determined by X-ray diffraction (XRD). A total of 24 months stability of the amorphous azithromycin state in the silica (33.2 % loading) and 18 months stability in the gel (33.2 % loading) at 4 °C were also confirmed by XRD. The higher kinetic solubility at 40 min (1,300 µg/mL versus 93 µg/mL) and higher porcine ear skin penetration compared to the raw drug powder indicated higher dermal bioavailability of the azithromycin-loaded silica (32.0 % loading), even when compared to the "gold standard" nanocrystals and another clinical effective azithromycin formulation with ethanol. In summary, maximum loaded silicas with azithromycin by WIFS is a promising dermal formulation for prophylaxis against Lyme disease.

1. Introduction

Humans have been suffering from Lyme disease for more than a century in North America and Europe, which occurs when an infection is ignored after a tick bite (Weber 2001; Wormser et al. 2006). This disease can lead to very serious symptoms and can be life-threatening (Shapiro 2014). Stays in woodlands and contact with dogs or cats ensures that tick attachment occurs more frequently. The best methods for preventing Lyme disease are vaccines and prophylactic antimicrobial therapy (Piesman et al. 2014). However, a vaccine to prevent Lyme disease in humans in currently unavailable (Piesman et al. 2014). Thus, the only potential prevention method is the administration of antimicrobial agents. The typical treatment regimens include doxycycline, amoxicillin, and cefuroxime axetil (Shapiro 2014). However, their oral long-term treatment causes inconvenience to patients and side effects such as vomiting, nausea, and rashes (Knauer et al. 2011). Besides, bacterial resistance to the administered antibiotic or malaise toxicity have been shown after liberal oral administration of antibiotics (Venkatesh et al. 2013). Thus, topical treatment is an option. Topical applications of antibiotics were studied and proven to bee effective for Lyme disease already in 1993 (Shin and Spielman 1993); however, study authors did not recommend their formulation for human use because dimethyl sulfoxide (DMSO) was used as the solvent in the carrier. Thus, a novel dermal formulation for hydrophobic antimicrobial agents without organic solvents is a demand. Azithromycin was selected as model drug in present study owing to its lower minimum inhibitory concentration, superior activity against *Borrelia burgdorferi,* fewer adverse effects (Knauer et al. 2011; Weber et al. 1993), and quicker onset time (Weber et al. 1993). Most importantly, a dermal azithromycin formulation has been proven to possess higher potency than doxycycline following a tick bite in a murine model system (Piesman et al. 2014).

Converting crystalline active pharmaceutical ingredients (APIs) to their amorphous counterparts is one advocated strategy to enhance the bioavailability of hydrophobic APIs without organic solvent. This is because APIs in their amorphous state lack crystal structures, resulting in less bonds. Thus, molecules in an amorphous state possess high molecular mobility and free energy (Löbmann et al. 2012; Yani et al. 2016), which leads to improved wettability (Badu and Nangia 2011). Consequently, the kinetic solubility of amorphous forms is higher than the corresponding solubility of crystalline drugs (Byrn et al. 1994; Wei et al. 2015). This high kinetic solubility generates a high concentration gradient between dermal formulation and the skin, thus increasing the diffusive flux into the skin. Therefore, the penetration of the poorly soluble drug is enhanced and results in high dermal bioavailability (Müller et al. 2011). However, a highly-energized amorphous state also results in re-crystallization during production, storage, or administration owing to less favorable thermodynamic conditions. Once the above alterations occur, the favorable advantages (increased solubility and dissolution) of the amorphous state disappear (Elder et al. 2013; Graeser et al. 2009; Laitinen et al. 2013).

Porous material has been considered as one alternative solution to yield the stable amorphization of poorly soluble APIs (Qian and Bogner 2012) for the following reasons. First, the low free energy state of the entire system maintains a long-lasting amorphous state (Qian and Bogner 2012). The unloaded porous material holds high surface free energy because of its large surface area. After the absorption of the drug molecules, the porous material will decrease the Gibbs free energy in the entire system. The lower free energy, in turn, will keep the amorphous drug physically stable for a long time (Williams et al. 2013). Second, the following unique properties lead to beneficial functions for loading drugs: 1) small pore size (Van Speybroeck et al. 2009) [micropore < 2 nm; 2 nm < mesopore (the most commonly uses) < 50 nm; macropore (seldomly used) > 50 nm] can restrict re-crystallization and drug nucleation because of spatial confinement (Qian and Bogner 2012; Salonen and Lehto 2008); 2) high specific surface area (500–1,500 m^2/g) is promising for the adsorption of agents (Prestidge et al.

2007; Wu et al. 2011); and 3) large pore volume (1.0–1.7 cm^3/g) and surface chemistries such as siloxane groups with oxygen (-Si-O-Si-) and silanol groups (-Si-OH) can be modified to control drug release (Rancan et al. 2012; Rigby et al. 2008; Simovic et al. 2011). The interaction between drug and functional groups of silicas can also lead to amorphization (Kinnari et al. 2011).

Among various porous materials, silica-based vehicles have been the most commonly investigated (Williams et al. 2013). Previous studies regarding the pharmaceutical application of porous silicas have mainly focused on oral drug delivery (Bimbo et al. 2011; Guo et al. 2013; Kinnari et al. 2011; Rigby et al. 2008; Van Speybroeck et al. 2009) and on implantable and injectable drug delivery (Salonen et al. 2008; Sarkar et al. 2016). However, little has been published regarding using porous silicas as vehicles to maintain drugs in the amorphous state for dermal preparations. Even if some research groups (Berlier et al. 2013a,b; Sapino et al. 2015; Scalia et al. 2013; Ugazio et al. 2016) have been interested in the dermal delivery of silicas, none have focused on drug amorphization. Some of these researchers only treated silica as a stabilizer or thickener of the topical formulation (Frelichowska et al. 2009; Rozman et al. 2010). In addition, their nanometric silica particles might increase the toxicity risk (Elle et al. 2016; Michel et al. 2013). The smartPearls® technology (Monsuur et al. 2014) used in the present study involves the transfer of the oral CapsMorph® technology (Wei et al. 2015), which can maintain the long physical stability of the amorphous state of the drug, to dermal applications. Both of these are the wetness impregnation method with fractional steps (WIFS) (Jin 2017). The carrier used in this technology are µm-range silicas with nano-range pores. This technology combines two main approaches of increasing the solubility of the active–size reduction to the nanoscale and drug amorphization (Kawabata et al. 2011; Monsuur et al. 2014). Therefore, it encages the amorphous active in the mesopores of the porous µm-sized particles with nm-sized pores (e.g., Davisil® in the present study). It also avoids high thermodynamic instability because of the restriction of re-crystallization resulting from the tiny spaces. Thus, the amorphous state with high free energy is not easy to convert to a low free energy crystal state, and the amorphous state can be maintained for at least three years (Wei et al. 2015). Additionally, the nano-range size increases the saturation solubility owing to the Ostwald-Freundlich and Kelvin equations (Mauludin et al. 2009; Pardeike et al. 2010). Finally, the penetration of the poorly soluble drug is enhanced and results in high dermal bioavailability (Müller et al. 2011).

The aim of the present study was to produce amorphous azithromycin by WIFS and to investigate the maximum loading and stability of the amorphous azithromycin in Davisil® SP53D-11920 with and without hydrogel. To further understand the basic mechanisms of this novel technology, the influence of the loading procedure was studied. In addition, kinetic solubility and the porcine ear skin model were studied to show the ability of the novel azithromycin dermal formulation to penetrate the skin. For comparison, raw drug powder, "gold standard" nanocrystals with a particle diameter of 189 nm, and an azithromycin ethanol solution gel (comparable to one tested during clinical phases) (Knauer et al. 2011; Schwameis et al. 2017) were tested during the penetration study.

2. Investigations, results and discussion

2.1. Theory of loading drugs onto Davisil® porous materials

The loading method depends on the capillary force generated by the tiny pores (in the present study, this was 6 nm pore diameter), which draw the drug solution into the voids of the pores (Wei et al. 2015), although inevitably a small portion will be distributed onto the silica surface. With the evaporation of the solvent, the drug precipitates. The molecules in the pores cannot convert to long-range molecular order from the disordered arrangement due to the confined space, thus they stay in the amorphous state, whereas the other component of the drug molecules on the surface might be re-crystallized during storage. Thus, theoretical calculation is required to ensure that the total volume of the drug solution in each spray is smaller than the total volume of the unfilled pores.

The details of the calculation are as follows: R is the weight ratio of solvent and drug, D (g/mL) is the density of this solvent, d (g/mL) is the density of the drug, and P (mL/g) is the pore volume of the silica. Before loading the drug solution, the silica was dried in a compartment dryer until it reached a constant weight. During the first loading step, with the silica weight of M_1 (g) and the drug needing to be loaded weight of m_1 (g), the volume of the drug solution was approximately $[(R+1)*m_1]/D$, which should be smaller than the volume of unfilled pores, $P*M_1$, to ensure that the drug solution can stay inside the pores as much as possible. The complete solvent evaporation is controlled by determination of weight loss. If the obtained production is in an amorphous state that has been confirmed by X-ray diffraction (XRD) or differential scanning calorimetry, then the next step follows. If not, then the drug solution is diluted and this step repeated until the drug in this step is in an amorphous state. The drug loading of this step is L_1, $L_1 = m_1/(m_1+M_1)$. For the number n^{th} step of loading, with the formulation weight $M_{(n)}$ (g) (n>1) and the drug needing to be loaded with weight of $m_{(n)}$ (g), then the volume of the drug solution is approximately $[(R+1)*m_{(n)}]/D$. This should be smaller than the volume of unfilled pores, which is now $M_{(n)}*(1–L_{(n-1)})*P – (M_n*L_{(n-1)}/d)$. If the production in this step is amorphous, then continue with the next step. If not, the drug loading is compared with the theoretical maximum loading $[M_1*P*d/(M_1*P*d+M_1)]$. If there is no big difference between these two data, then this step should be the final step of the formulation. If there is a big difference between these two data, then the drug solution is diluted and the steps repeated from the first step again until the drug in this final step is amorphous. The drug loading of this step is L_n, $L_n = (m_n+M_n*L_{(n-1)})/(m_n+M_n)$. In the present study, D was 0.789 g/mL, d was 1.18 g/mL, and P was 0.9 mL/g. The theoretical maximum loading of azithromycin in Davisil® SP53D-11920 was 51.5% w/w.

2.2. Maximum loading detected by XRD

Overloading was observed by the detection of crystalline peaks in the X-ray spectrum, indicating that a fraction of the drug in the silica was crystalline. As shown in Fig. 1, the main crystalline peak (the highest intensity) of azithromycin was at approximately 10°, the same as that reported by Zhang et al. (2010). The maximum amorphous loading of azithromycin in Davisil® was 32.0 % (w/w) (no crystalline peak) produced by three spraying steps of higher concentrated azithromycin-ethanol solution (c_s 50 %). Even though one more spraying step resulted in only 33.3% (w/w) loading, this sample showed overloading that was confirmed by XRD due to the sharp peak at approximately 10° (Fig. 1, left). The first spraying step and the second spraying step resulted in 16.7 and 27.4 % loading, respectively. For the lower concentrated azithromycin-ethanol solution (c_s 25 %), each spraying step resulted in less loading silica, but ended in a silica with maximum 33.2 % (w/w) loading (no crystalline peak) after seven spraying steps. Here, overloading was detected as 35.0 % (w/w) loading after eight spraying steps (Fig. 1, right, a sharp peak at approximately 10°). Thus, the different loading effects were determined by the different spraying solution concentrations. The less concentrated the spraying solution, the more maximum loading is approachable and the more spraying steps are needed. This might be because it is easier for the drug to enter the deep pores with the less concentrated spraying solution, resulting in more space for the next loading.

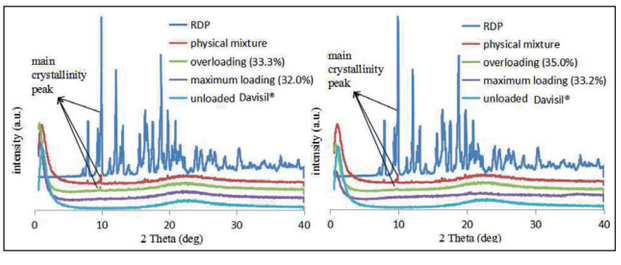

Fig. 1. X-ray spectrum (vertical axis: intensity; horizontal axis: 2 Theta) of maximum loading (left: high concentrated azithromycin-ethanol solution (c_s50%); right: low concentrated azithromycin-ethanol solution (c_s25%)).

2.3. Amorphous stability

The stability of the amorphous state in the Davisil® and in the gels was also detected by XRD because XRD is the most suitable method for studying the physical state of crystalline and amorphous formulations (Wei et al. 2015). The maximum loaded Davisil® with c_s 50 % showed much lower physical stability than that of the Davisil® maximum loaded with c_s 25 %. Its amorphous drug stayed stable for less than 1 week at room temperature (25-30 °C, RT) (Fig. 2, crystalline peak shown at day 7), whereas the maximum loaded Davisil® with c_s25% maintained its amorphous state until 15 months (Fig. 3, left, no crystalline peak at 14 months) and up to 24 months at 4 °C (Fig. 3, right, no crystalline peak at 24 months). When the maximum loaded Davisil® with c_s 25 % was incorporated into 5 % (w/w) hydroxypropyl cellulose (HPC) gel, the HPC gel base as the blank control (without drug or silica inside) showed a broad peak at approximately 30° in the XRD spectrum (Fig. 4). The physical mixture of raw drug powder and HPC gel showed a crystalline peak at approximately 10°, corresponding to the XRD spectra of the raw drug powder. However, the azithromycin (AZ)-Davisil gel showed no crystalline peak at 1 day, 6 months, and 18 months when stored at 4 °C (Fig. 2, right). Therefore, the amorphous state of the 33.2 % (w/w) azithromycin-loaded Davisil® could be stabilized for at least 18 months even in the water-containing medium at 4 °C. Usually, amorphous solid formulations store potential energy, which will release in contact with a liquid such as water (Wei et al. 2015). In that case, the amorphous molecules will generate an unstable supersaturated state, resulting in re-crystallization and formation of crystal nuclei occurs (Brauwers et al. 2009). This makes it difficult to develop a dermal system in amorphous state (Monsuur et al. 2014). However, the XRD results indicated that the Davisil® system not only possesses the potency of long physical stability of the amorphous state, but also makes it possible to incorporate the amorphous solid formulation into dermal gels.

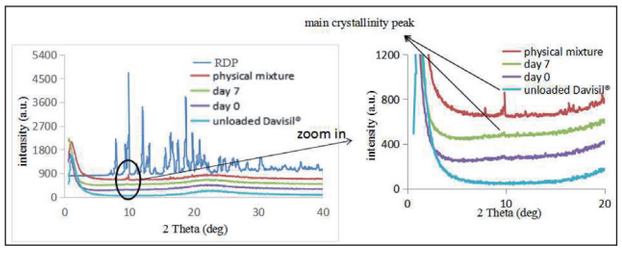

Fig. 2: X-ray spectrum (vertical axis: intensity; horizontal axis: 2 Theta) of maximum loaded Davisil® produced by high concentrated azithromycin-ethanol solution (c_s50%) stored at RT.

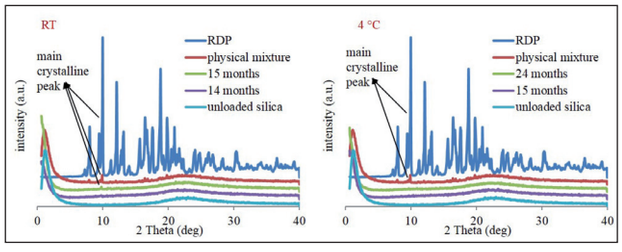

Fig. 3: X-ray spectrum (vertical axis: intensity; horizontal axis: 2 Theta) of maximum loaded Davisil® produced by low concentrated azithromycin-ethanol solution (c_s25%) (left: at RT; right: at 4 °C).

2.4. Saturation solubility studies

The kinetic solubilities are shown in Fig. 5. The kinetic solubilities of the raw drug powder did not changed to any great amount within 1 h. The kinetic solubilities of the physical mixture were lower than those of the raw drug powder before 20 min, but higher than those of the raw drug powder after 20 min. This may be due to spontaneous amorphous transformation when the crystalline raw drug powder

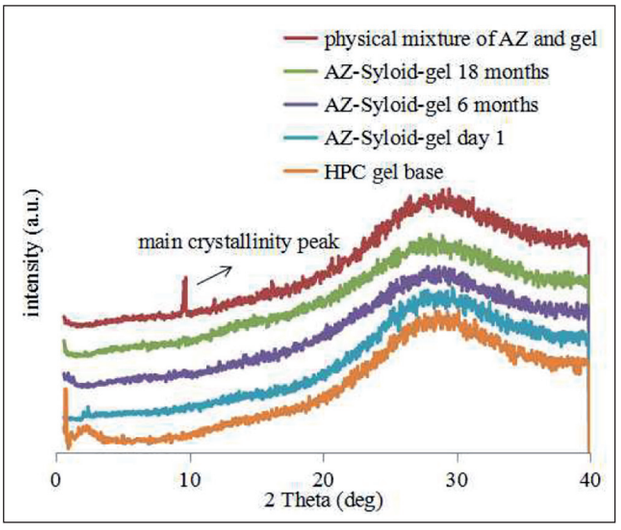

Fig. 4: X-ray spectrum (vertical axis: intensity; horizontal axis: 2 Theta) of 33.2% azithromycin-loaded Davisil® incorporated into 5% HPC gel stored at 4 °C for 18 months.

was physically mixed with the mesoporous materials (Qian 2011). However, when observing the slightly enhanced solubilities of the physical mixture with increasing time, there was a huge difference between the kinetic solubilities of the physical mixture and those of the loaded silica, i.e., it showed six times lower solubilities (213 µg/mL) than that of the loaded silica (1,300 µg/mL) in 40 min. This confirmed that the loaded silicas produced by WIFS had a stronger ability to amorphize azithromycin compared to physical mixing. The kinetic solubility of the 32.0 % (w/w) loaded Davisil® after 40 min was 1,300 µg/mL, which was almost 14 times higher than that of the raw drug powder (93 µg/mL). This high solubility implied the potentially high skin penetration of the azithromycin-loaded Davisil®.

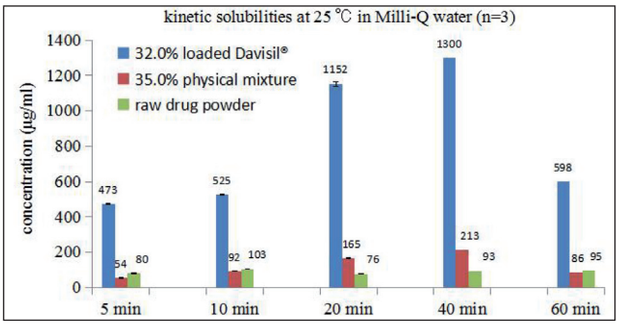

Fig. 5: Kinetic solubilities of the 32.0 % (w/w) azithromycin-loaded Davisil®, the physical mixture (unloaded Davisil® and raw drug powder) and the raw drug powder at 25 °C in Milli-Q water as a function of time (n = 3).

2.5. Penetration study with porcine ear skin

Porcine ear skin *ex vivo* is a valid model to test the penetration potency of its human counterpart (Sekkat et al. 2002). When comparing data between different formulations in the porcine ear penetration study, the first tape was excluded from the analysis because it was only occupied by the non-penetrated drug. Data in Fig. 6 are expressed as the normalized penetrated drug amount (µg/%) per cm^2 *versus* the tape numbers. The tape number was correlated with the depth of skin penetration, where higher tape numbers represent deeper penetration. The AZ-Davisil gel demonstrated the highest penetration efficiency of the amorphous azithromycin, having up to 30 times higher active concentration in the deep skin layers (tape 20-30) than that of the raw drug powder. In contrast, the ethanol solution gel stayed mainly close to the surface (tape 2-7), showing approximately three times higher penetration

than that of the raw drug powder, but only half the penetration efficiency compared to the AZ-Davisil gel in tape 2-7 and even less in other deeper skin agers. The delivery efficiency of the AZ-Davisil gel in all the tested tapes was higher than the "gold standard" nanocrystals (particle diameter 189 nm). This phenomenon might be due to both the re-crystallization from the solution formulation (supersaturation sate of azithromycin in ethanol was thermodynamically unstable) (Moser et al. 2001; Steele 2009) and the increased kinetic solubility of the amorphous azithromycin in Davisil® mentioned in section 2.4. According to Fick's first law of diffusion, increased solubility leads to increased concentration gradient between the *stratum corneum* and the vehicle, and thus results in an enhanced drug flux (Tam et al. 2008; Watkinson et al. 2009; Zhai et al. 2014).

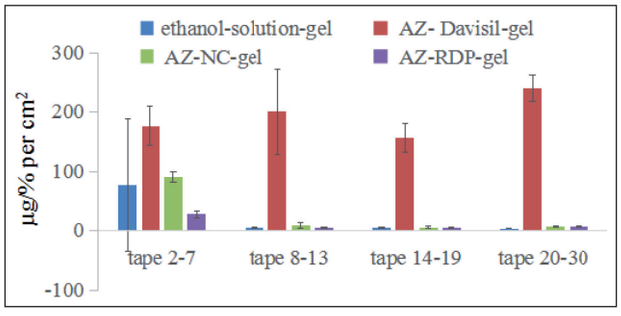

Fig. 6: Normalized amount of azithromycin in tapes after 20 minutes penetration, calculated by dividing the amount of drug (µg) by the azithromycin concentration (%) in the applied formulation per cm^2 (n = 3).

2.6. Conclusions

When WIFS was applied, one maximum azithromycin loading (33.2 % loading) was achieved using azithromycin-ethanol solution (c_s 25 %) after seven loading steps. Another maximum azithromycin loading (32.0 % loading) was achieved using azithromycin-ethanol solution (c_s 50 %) after three loading steps. This maximum loaded Davisil® produced with the high concentrated azithromycin-ethanol solution (c_s 50 %) was much less stable than that produced with the low concentrated azithromycin-ethanol solution (c_s 25 %). The 33.2 % loaded amorphous azithromycin could be loaded stably for the long-term onto Davisil® for 24 months at 4 °C, as well as being maintained in its amorphous state in water-containing HPC gel for at least 18 months. Moreover, the azithromycin-loaded Davisil® showed much higher kinetic solubility than the raw drug powder, the "gold standard" azithromycin nanocrystals with particle diameter 189 nm, and the physical mixture of raw drug powder and unloaded silicas, indicating a high dermal bioavailability, which was also confirmed by the *ex vivo* penetration study. It had a superior efficacy of dermal penetration compared to the clinical effective ethanol solution gel even at low active concentrations.

Therefore, WIFS is attractive to generate a long-term stable amorphous azithromycin dermal formulation with increased skin penetration. This would enable amorphous azithromycin to be exploited for topical administration with a high dermal bioavailability for a high prophylactic effect against Lyme disease. It is also industrially feasible because suitable porous microparticles can be purchased on the market. The 10–40 µm sized particles of the selected silicas also avoid the controversy of nanotoxicity. In summary, applying the azithromycin-loaded Davisil® gel at the site of tick exposure immediately after a tick bite should decrease the risk of acquiring Lyme disease infection.

3. Experimental

3.1. Materials

Azithromycin dihydrate (contains 95.4% azithromycin) was ordered from Haohua Industry (Jinan, China). Klucel GF® (hydroxypropyl cellulose) purchased from Caesar & Loretz GmbH (Hilden, Germany) was used as gelling agent. Davisil® SP53D-11920 (Davisil®) was kindly provided by W. R. Grace & Co. (Worms, Germany). Ethanol (96%) used of was analytical grade. Milli-Q water was obtained by reverse osmosis from a Millipak® Express 20 Filter unit (Merck, Germany).

3.2. Production of smartPearls® – maximum loading of azithromycin

The maximum dissolvable amount of azithromycin in 10.0 g ethanol (96 %) was approximately 5.0 g, representing a saturation solubility c_s in ethanol (96 %) of 50 % (w/w). Two drug-ethanol solutions were prepared for drug loading: one with an azithromycin concentration half that of c_s (c_s 50 % = 5.0 g azithromycin in 20.0 g ethanol (96 %)) and the other with one quarter that of c_s (c_s 25 % = 5.0 g azithromycin in 40.0 g ethanol (96 %)). Each solution was then sprayed stepwise by a spraying nozzle screwed onto a glass bottle to certain amounts of dried silica. The silica was stirred using an ointment bowl and pestle after every spray to ensure that the drug solution was immediately and homogenously absorbed by the silica. Each spray was loaded with approximately 0.1 g drug-ethanol solution. After finishing spraying a certain amount of the solution, the sample was moved to a flat-bottomed glass vessel and stored in a compartment dryer at 40 °C until all the ethanol had evaporated. This evaporation process was controlled via the weight loss technique. After cooling to RT, the amorphous state of the sample was monitored by XRD. Subsequently, these steps were repeated until the maximum loading was reached. The details of the loading data in each step are shown in the Table.

3.3. Preparation of azithromycin gels

For the preparation of the gel base, Milli-Q water was heated to 75 °C in an ointment bowl. Subsequently, the HPC powder was added to the water and dispersed using a pestle until a homogenous suspension resulted. The mixture was placed into a transparent gel base after storage overnight at 4 °C in the refrigerator. Overnight evaporated Milli-Q water was supplemented at RT. A weighted amount of the two maximum azithromycin-loaded Davisil® were incorporated into the 5.0 % (w/w) HPC gel to obtain a final concentration of 1.0 % amorphous azithromycin in the gel (AZ-Davisil gel). A total of 5.0 % raw drug powder and azithromycin nanocrystals with particle diameter of 189 nm produced by our group were incorporated into the gel to obtain a 5.0 % azithromycin raw drug powder gel (AZ-raw drug powder gel) and a 5.0% azithromycin nanocrystals gel (AZ-NC gel). A 10.0 % ethanol solution gel (10.0 % azithromycin raw drug powder, 77.5 % ethanol (94 %), 0.5 % polyacrylate, 5.0 % HPC, and 7.0 % Miglyol® 812) was selected as a comparison, which demonstrated the effectiveness of the clinical studies (Knauer et al. 2011; Schwameis et al. 2017).

3.4. Determination of solid state

A PW 1830 X-ray diffractometer (Philips, Netherlands) was used to monitor the solid state of azithromycin loaded in Davisil® and in gels. Overloading was observed by detecting crystalline peaks (sharp peaks) in the X-ray spectrum, which means that

Table: Details of loading data of azithromycin onto silicas in each step

	32.0 % (w/w) final loading			33.2 % (w/w) final loading		
Concentration of drug-ethanol solution	c_s 50 %			c_s 25 %		
	Obtained silica from the last step (g)	Drug-ethanol solution (g)	Drug loading (%)	Obtained silica from the last step (g)	Drug-ethanol solution (g)	Drug loading (%)
Step 1	10.00	10.00	16.7	10.00	9.00	9.1
Step 2	9.00	8.00	27.4	8.00	5.40	14.9
Step 3	8.10	3.72	32.0	6.00	4.32	20.3
Step 4	/			4.80	2.88	24.3
Step 5	/			3.60	2.16	28.0
Step 6	/			2.88	1.12	30.7
Step 7	/			2.30	1.08	33.2

the drug was not completely in the amorphous state. The samples were analyzed by placing a thin layer of the loaded silica powder or gels in the X-ray diffractometer. The diffraction angle range was between 0.6° and 40° with a step size of 0.04° per 2 s. The diffraction pattern was measured at a voltage of 40 kV and a current of 25 mA.

3.5. Determination of the kinetic solubility

For kinetic solubility studies, the 32.0 % (w/w) azithromycin-loaded Davisil® was dispersed in Milli-Q water to obtain a final concentration of azithromycin of 4.8 % in vials. Azithromycin and unloaded Davisil® physical mixture (35.0 % azithromycin) were also dispersed in Milli-Q water to obtain a final concentration of azithromycin of 5.6 % in vials. Furthermore, 5.6 % raw drug powder was suspended in Milli-Q water for comparison. The samples were stored at 25 °C and shaken at 100 rpm in an Innova 4230 shaker (New Brunswick Scientific GmbH, Nürtingen, Germany) for 60 min. To separate the dissolved drug, the samples were first centrifuged (17,968 × *g*) at RT for 10 min and the supernatant was subsequently filtered with nucleopore track-etched polycarbonate membrane filters (50 nm pore size, Whatman® 110603 filter). The drug concentration in the obtained samples was determined by high performance liquid chromatography (HPLC).

3.6. Penetration study with porcine ear skin

A penetration study via tape stripping was performed in the porcine ear skin model as follows: 100 mg AZ-Davisil gel with 1.0 % amorphous azithromycin, 100 mg AZ-raw drug powder gel with 5.0 % azithromycin raw drug powder, 100 mg AZ-NC gel with 5.0 % azithromycin nanocrystals, and 100 mg ethanol solution gel with 10.0 % azithromycin dissolved in ethanol were applied homogenously onto a skin area of 1.5 × 1.5 cm^2. After a penetration time of 20 min, adhesive tape was pressed on to the skin using a roller and then rapidly removed. One area was taped 30 times. Afterwards, the drug was quantitatively extracted from the tape strips using 2 mL of acetonitrile/DMSO (1:1, v/v) as solvent and shaken for 3 h at 120 rpm in an Innova 4230 shaker. Subsequently, the samples were centrifuged (15,493 × *g*; 15 min) and the supernatant was analyzed by HPLC as described in section 3.7.

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). A Lichrospher-100 RP-8 reverse phase column (125 × 4 mm, 5 µm) was used. For the 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 K_2HPO_4 and H_3PO_4 at pH 7.5. The column temperature was set to 45 °C. The UV detector measured azithromycin at a wavelength of 210 nm.

Conflicts of interest: None declared.

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