The system SLN-Dextran hydrogel: An application for the topical delivery of Ketoconazole

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ABSTRACT
Ketoconazole (KTZ) is a widely used antifungal drug. Despite its broad spectrum as antifungal agent, it suffers from two characteristics that make difficult its utilization. It is poorly water soluble and undergoes very easily chemical degradation, such as oxidation and hydrolysis. The paper reports a new system based on solid lipid nanoparticles (SLN) entrapped into polysaccharidic hydrogels to be employed as modified delivery system of ketoconazole in topical formulations. Three different lipid phases (compitol, precirol and a mixture precirol/almond oil) were employed to prepare SLN and all the nanoparticles were characterized for their mean particle diameter, polydispersion index and zeta potential. All the systems were able to efficiently encapsulate the drug (entrapment efficiency > 90%). SLN formulations were able to protect the drug from UV degradation, even if partially. The incorporation of KTZ-loaded SLN into dextran hydrogels allowed the preparation of systems having rheological characteristics suitable for different topical applications. The efficacy of the system has been exploited testing the antifungal activity against Candida albicans, whereas the skin tolerability of all the preparation components was tested on rabbits.

Keywords: Solid lipid nanoparticles; Dextran methacrylate hydrogels; Ketoconazole; Modified release; Candida albicans.

INTRODUCTION
In recent years a remarkable increase of fungal infections has taken place. Opportunistic infections, such as aspergillosis, candidiasis, and cryptococcosis, have emerged as major problems in cancer patients, transplant recipients, and other immunocompromised individuals,
including those with AIDS [1-3]. Vulvovaginal candidiasis is a common mucosal infection affecting many women, with several case of recurrence and therapeutic failure, up to 75% of all women experience at least one episode during their life [4]. *Candida albicans* is the principal responsible accounting for over 80% of total vulvovaginal candidiasis.

The antifungal pharmacotherapy was revolutionized since the Nineties by the introduction of azolic drugs. Ketoconazole (KTZ), cis-1-acetyl-1-4-[4-2-(2,4-dichlorophenyl)-2-2-(1H-imidazole-1yl-methyl)-1,3-dioxolan-4-yl]methoxy piperrazine, is a broad spectrum agent used in the treatment of systemic and superficial fungal infections [5]. Recently, new formulations of KTZ have been brought to the market for the treatment of seborrheic dermatitis, giving a new life to its topical use [6]. KTZ shows limited water solubility; moreover this compound is not very stable because it undergoes chemical degradation, such as oxidation and hydrolysis, easily [7]. Solid lipid nanoparticles (SLN) represent an interesting approach to address both solubility and stability problems of KTZ. SLN are an efficient colloidal carrier system for the modified delivery of lipophilic drugs [8-10]. They are composed of physiological lipids and for their high biocompatibility find application in cosmetic and dermatological preparations [11] as well as in parenteral and oral drug formulations [12-16]. Furthermore this type of systems can offer effective protection to light- and/or water-sensitive drugs. Recently some attempts were made to load KTZ into SLN or NLC in order to improve its stability [17,18]. We have already proposed a new system constituted by solid lipid nanoparticles (SLN) entrapped into a chemical dextran-based hydrogel for the controlled delivery of lipophilic drugs in oral formulations [19] as well as in topical ones [20]. The model lipophilic drug (S)-(+-)2-(4-isobutylphenyl)-propionic acid (ibuprofen) was used to test the system.

We now planned to investigate the possibility of employing the SLN-hydrogel system to formulate KTZ. Two lipids with different melting points, added or not with almond oil were used to prepare the SLN and the obtained nanoparticles were characterized for their physical-chemical properties. Then SLN were entrapped into chemical polysaccharidic hydrogels and the release profiles of ketoconazole were studied. Moreover the SLN-loaded hydrogels were submitted to rheological measurements in order to assess their possible employment as topical formulations, whereas their antifungal activity was evaluated in vitro against *Candida albicans*. Finally primary skin irritation studies of all the components of the formulation were performed on rabbits.

**EXPERIMENTAL SECTION**

**2.1. Materials**

All the used reagents were of analytical grade. Dextran (DEX) from *Leuconostoc* ssp. (Mr 40000), dimethylsulfoxide (DMSO), glycicyl methacrylate (GMA), 4-dimethylaminopyridine (DMAP), ammonium peroxydisulphate (APS), N,N,N',N'-tetramethylethylendiamine (TEMED) and cholic acid sodium salt were purchased from Fluka (Switzerland), Sephadex 75-G from Sigma (USA), D₂O from Aldrich Chemical (USA), Lutrol F68 (poloxamer 188) from BASF (Germany). Ketoconazole (KTZ) was a kind gift of Janssen (Italy). Precirol® ATO 5 and Compritol® 888 ATO were kindly provided as a gift by Gattefossé (France). Precirol® ATO 5 consists of a mixture of 40% tri-, 45% di- and 14% monoglycerides of palmitic and stearic acids. Compritol® 888 ATO is a mixture of 13-21% mono-, 40-60% di- and 21-35% triglycerides of palmitic, stearic, arachidic, behenic, erucic and lignoceric acids, with behenic acid as principal component accounting for more than 83% of the total fatty acids. Pure almond oil was purchased from Agrar (Italy). Dialysis membranes (cut-off 12,000-14,000) were from Medicell International (U.K.). *Candida albicans* ATCC 24433 was obtained from American type culture
collection (ATCC, Rockville, MD, USA). RPMI 1640, MOPS and sabouraud dextrose agar were purchased from Sigma-Aldrich (USA).

2.2. Synthesis of dextran methacrylate (DEX-MA)
Dextran methacrylate was synthesized as already reported [21]. 4-DMAP (2.0 g) and GMA (1.5 g, 2mol / 6mol of repetitive unit) were added to a solution of dextran (5.0 g) in DMSO (40 ml). The solution was maintained under stirring at room temperature for 24 h. The polymer was precipitated with EtOH (200 ml), recovered by filtration, dissolved in water (15 ml) and the solution, adjusted to pH 8 with 1.0 M HCl, was submitted to exhaustive dialysis. After freeze-dried, the polymer was characterized by FT-IR, $^{13}$C- and $^1$H-NMR. FT-IR spectra were recorded with a Perkin Elmer Paragon 1000 spectrophotometer in the range 4000-400 cm$^{-1}$ using KBr pellets (resolution of 1 cm$^{-1}$). $^1$H-NMR spectra were obtained with a Bruker AC-400 instrument. The degree of derivatization (amount of methacryloyl groups for 100 glucopyranosyl residues, DD), calculated on the basis of the $^1$H-NMR spectrum, as reported in the literature [21,22], was 40 ± 1. DEX-MA having DD=20 ± 1 was synthesized with the same procedure, using 1.0 g of 4-DMAP and 0.73 g of GMA for 5.0 g of starting polymer.

2.3. Preparation of solid lipid nanoparticles (SLN)
Solid lipid nanoparticles were prepared with the hot homogenization technique [23]. Five g of lipid [Precirol$^{\text{\textregistered}}$ ATO 5, Precirol$^{\text{\textregistered}}$ ATO 5/almond oil 85:15 (w/w) or Compritol$^{\text{\textregistered}}$ 888 ATO] were heated at a temperature at least 10 °C above their melting point (55.2, 54.5 and 73.2°C respectively). When completely melted, the lipid phase was added with an aqueous solution (45 ml) of the surfactants (sodium cholate, 0.50 g and poloxamer, 1.25 g), heated at the same temperature [24]. The mixture was homogenized with an Ultra-Turrax T18 Basic (IKA-WERK, Germany) for 10 minutes at 24000 rpm and then left to cool down to room temperature. Drug loaded SLN were prepared by adding KTZ (0.20 g) to the lipid phase and following the previously described procedure.

Samples were labeled as SLN$_P$, NLC$_{PO}$, SLN$_C$, SLN$_{PK}$, NLC$_{POK}$ and SLN$_{CK}$, where P, O, C and K stand for Precirol$^{\text{\textregistered}}$ ATO 5, almond oil, Compritol$^{\text{\textregistered}}$ 888 ATO and ketoconazole respectively.

2.4. Particle size analysis and zeta potential measurements
The particle size analysis of all SLN samples was performed by photon correlation spectroscopy (PCS) immediately after the preparation. The PCS analysis yielded the mean diameter of the particles (Z-average) and the polydispersity index (PDI), as a measure of the width of the particle size distribution. The samples were properly diluted with twice distilled water and analyzed with a Malvern Zetasizer nano ZS90 (Malvern Instruments, UK). The surface charge of all the samples was determined by measurements of the zeta potential carried out with the same instrument.

2.5. Differential scanning calorimetry (DSC) measurements
DSC measurements were carried out with a SETARAM DSC 131 (France), equipped with SETSOFT 2000. The samples were quickly frozen in liquid nitrogen and freeze-dried at room temperature and pressure of 0.4-0.5 Hg mm. All freeze-dried samples were left under vacuum on P$_2$O$_5$ for 3 days before the DSC investigation was carried out, in order to ensure the complete elimination of the moisture. Exactly weighed samples (10 mg) were sealed in standard aluminium pans (30 µl) and submitted to calorimetric analysis at a scan rate of 10°C/min in the temperature range 15-160°C, using an empty pan as reference. Analyses were performed under nitrogen purge.
2.6. Determination of encapsulated ketoconazole
The amount of KTZ encapsulated into the three SLN systems was determined by extracting the drug from the nanoparticles after separation of the un-encapsulated KTZ. To this end, aliquots of each SLN preparation were quickly passed through a column of Sephadex 75-G, employing distilled water as eluant. The recovered nanoparticles were lyophilized and the residues extracted with methanol. The amount of the drug was determined by means of HPLC analysis, monitoring at 240 nm. HPLC apparatus consisted of a Perkin Elmer Series 200 LC pump, equipped with a 235 Diode Array Detector. HPLC analyses were carried out using a Merck Hibar LiChrocart (250-4, 5µm) RP-18 column, with a flow of 1 ml/min. A mixture of aqueous diethylamine (1% w/w) and methanol (20:80) was used as eluant for the analyses. All the experiments were performed in triplicate and the results reported as mean values ± S.D.

2.7. Ketoconazole stability to UV irradiation
KTZ (20.0 mg) was dissolved in MeOH (5 ml) and submitted to UV irradiation performed with a Helios Italquartz Photochemical Multirays Reactor (Italy) equipped with ten, 14 watt medium pressure, mercury lamps (λ maximum 310 nm) for 4 h. At pre-established time intervals (1, 2, 3 and 4 h), aliquots of the KTZ solution were withdrawn and the amount of residual drug was determined by means of HPLC analysis, as previously described. Same experiments were performed on SLNPK, NLCPOK and SLNCK samples. After the irradiation, the nanoparticles samples were freeze-dried, the residues were extracted with MeOH and analyzed as already described.

2.8. Hydrogels preparation
DEX-MA (DD=20 and 40, 1.25 g, 2.5% w/w) was added to all the SLN samples. The cross-linking reaction of DEX-MA was carried out adding appropriate amounts of APS (0.77 mmol APS/mmol methacrylic groups) and TEMED (0.2 mmol) to 10 ml of each preparation. In the same way, DEX-MA20 hydrogels with a polymer content of 3.75 and 5% w/v were also prepared by free radical polymerization.

2.9. Drug release studies
Drug release experiments were performed on fresh SLN-dextran hydrogels. Samples of the formulations (0.05g) were placed in 50 ml of acetate buffer (pH=4.0) and maintained under stirring at 37.0 ± 0.1°C for 8 hours. At appropriate times, 0.5 ml of release medium were withdrawn and replaced with the same amount of fresh medium. The release of the drug was followed by means of HPLC analysis, as described before. All the experiments were carried out in triplicate and the results were reported as mean values ± SD.

2.10. Rheological measurements
Rheological experiments were performed with a Haake RheoStress 300 Rotational Rheometer (Germany) equipped with a Haake DC10 thermostat. Oscillatory experiments were performed on all the SLN suspensions, prepared with the different lipid phases. Mechanical spectra were also recorded after addition of DEX-MA and cross-linking reaction. Enough quantity of each sample was carefully poured to completely cover the 6 cm cone-plate geometry (angle of 1°). Frequency sweep experiments were performed on all the samples at 25.0 ± 0.2°C in the range 0.01–10 Hz, in the linear viscoelastic region, assessed by preliminary stress sweep experiments. The mechanical spectra were then recorded applying a constant deformation in the linear region: usually a 1% maximum deformation was used.
2.11. Microbiological studies
The activity of samples of dextran hydrogel entrapping SLN\(_k\) was tested against \textit{C. albicans} ATCC 24433, recommended as reference strain in CLSI standard protocol [25] and compared with a commercial cream (Nizoral 2%, J&J) and a ketoconazole solution. Experiments were performed as previously described with some modifications [26]. Experiments were carried out in RPMI 1640 medium at two different pH values (7.0 and 5.0). RPMI 1640 was prepared according to CLSI standard [25] and medium was buffered to pH 7.0 or 5.0 with MOPS. For ketoconazole solution, the drug was first dissolved in DMSO at a concentration 100 times higher than the test concentration, as recommended by CLSI [27] and then diluted in buffered medium at a concentration of 50 µg/ml. At used drug concentration the solvent did not interfere with cell viability. Samples of SLN\(_k\) DEX-MA hydrogel, commercial cream and ketoconazole solution, containing 50 µg/ml of drug, were inoculated with \textit{C. albicans} cells suspension adjusted to attain a final concentration of \(1 \times 10^6\) cells/ml and incubated at 37°C for 4, 6, 24 and 48 h. Suspensions were then appropriately diluted in saline, spread by inclusion in sabouraud dextrose agar and incubated at 37°C for 24 h; Colony-Forming Units were then quantified. The experiments were performed in triplicate on three consecutive test days.

2.12. In-vivo skin tolerability studies
Three healthy male New Zealand White rabbits were purchased from Charles River (Calco, Lecco, Italy) and acclimated in the laboratory for a week. The rabbits were individually housed and received standard diet and tap water \textit{ad libitum}. Principles of laboratory animal care (EEC Directive of 1986; 86/609/EEC) guidelines were followed. The back of the animals was clipped free of fur with an electric clipper 24 h before application of the samples. The clipped areas of the skin were divided into four parts of same area (30×30 mm). DEX-MA hydrogel and the systems DEX-MA hydrogel/SLN\(_P\) (loaded or not with KTZ) were applied to three sites (approximately 500 mg/site); whereas the last part was used as a control. The treated and the control sites were covered by gauze and the back of the rabbit was wrapped with a non-occlusive bandage, thereafter the animals were returned to their cages. After 4 h, the bandage and the test material were removed and 1 h later the sites were examined for skin irritation. Observation of the sites with material and control was repeated after 24, 48 and 72 h. The reaction, defined as erythema (Er) or edema (Ed), was evaluated according to the score of the skin reactions already reported [28]. The Score of Primary Irritation (SPI) was calculated for each rabbit as the difference between the sum of the scores for erythema (Er) and edema (Ed), at 24, 48 and 72 h divided by the number of the observations for the treated sites and the sum of the scores for erythema (Er) and edema (Ed), at 24, 48 and 72 h divided by the number of the observations for the control sites, according to the formula:

\[
\text{SPI} = \frac{\sum (\text{Er} + \text{Ed})_{24h} + (\text{Er} + \text{Ed})_{48h} + (\text{Er} + \text{Ed})_{72h}}{N. \text{ observations}_T} - \frac{\sum (\text{Er} + \text{Ed})_{24h} + (\text{Er} + \text{Ed})_{48h} + (\text{Er} + \text{Ed})_{72h}}{N. \text{ observations}_C}
\]

with \(T\), treated; \(C\), control; \(Er\), erythema; \(Ed\), edema.

The Primary Irritation Index (PII) was calculated as the arithmetical mean of the SPI values of the three animals. The evaluation of PII was performed according to the categories reported in the literature [28].
RESULTS AND DISCUSSION

3.1. Preparation and characterization of solid lipid nanoparticles (SLN) samples

Solid lipid nanoparticles were prepared with the hot homogenization procedure already reported [23]. All the samples were analyzed for their mean particle size, size distribution and zeta potential value and the results are reported in Table 1.

Table 1. Physico-chemical characteristics of SLN. Mean diameter (Z-average), polydispersity index (PDI), zeta potential and entrapment efficiency values of the SLN formulations obtained with different lipid phases (mean value ± SD, n=3), containing or not ketoconazole.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean diameter (nm)</th>
<th>PDI</th>
<th>Zeta potential (mV)</th>
<th>Entrapment Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLN_P</td>
<td>148 ± 3</td>
<td>0.281 ± 0.008</td>
<td>-26.8 ± 1.0</td>
<td>-----</td>
</tr>
<tr>
<td>SLN_PK</td>
<td>132 ± 2</td>
<td>0.270 ± 0.030</td>
<td>-33.6 ± 1.9</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>SLN_C</td>
<td>202 ± 1</td>
<td>0.212 ± 0.010</td>
<td>-31.7 ± 0.7</td>
<td>-----</td>
</tr>
<tr>
<td>SLN_CK</td>
<td>266 ± 3</td>
<td>0.234 ± 0.012</td>
<td>-23.3 ± 1.4</td>
<td>94 ± 3</td>
</tr>
<tr>
<td>NLC_PO</td>
<td>131 ± 3</td>
<td>0.104 ± 0.007</td>
<td>-36.4 ± 1.1</td>
<td>-----</td>
</tr>
<tr>
<td>NLC_POR</td>
<td>156 ± 2</td>
<td>0.177 ± 0.006</td>
<td>-30.4 ± 1.7</td>
<td>95 ± 2</td>
</tr>
</tbody>
</table>

In general, the average particle diameter of the SLN dispersions increases with the melting point of the lipids, because of the higher viscosity of the dispersed phase [23]. In fact the average size of SLN_P was found to be significantly smaller (148 ± 3 nm) than the size of SLN_C (202 ± 4 nm). In accordance with this hypothesis the addition of an oil to Precirol® ATO 5 gave rise to particles with an average diameter of 131 ± 3 nm. In all the cases the values of polydispersity index (PDI), a measure of the width of the particle size distribution, were lower than 0.3 so indicating a good homogeneity of the dispersions. Furthermore the surface charge of the particles was always negative and the values were high enough to assure a good stability of the nano-suspensions.

DSC experiments were also carried out on the components of the preparations (Table 2).

Table 2. Calorimetric studies. DSC melting peak data of SLN_C, SLN_P and SLN_POR samples.

<table>
<thead>
<tr>
<th>Melting peak (°C)</th>
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<tbody>
<tr>
<td>Bulk lipid</td>
</tr>
<tr>
<td>Compritol® ATO 888</td>
</tr>
<tr>
<td>Precirol® ATO 5</td>
</tr>
<tr>
<td>Precirol® ATO 5/almond oil</td>
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</tbody>
</table>

The melting point of bulk Compritol® 888 ATO is 73.2°C. When this lipid is formulated as SLN, the value of the melting point decreases to 72.9°C, probably for the presence of the surfactants. In the sample of SLN containing KTZ (SLN_CK), the melting point decreases till 71.4°C showing that an interaction between the lipid phase and the drug (having melting point 153.5°C) has taken place and that the latter is at least in part loaded into the particles. This behavior is already described in the literature for similar systems [29,30]. Analogous results were obtained for the formulations SLN_P and NLC_POR. Even in these cases the formulation of the lipids as nanoparticles
caused a shift of the melting peak, which was further modified by the incorporation of the drug, thus indicating the occurrence of an interaction between the lipid matrix and KTZ.

The amount of KTZ encapsulated into each SLN sample was determined by extracting the drug from the nanoparticles, after separation of the un-entrapped KTZ by gel filtration chromatography. The results show very high entrapment efficiency for all the systems and a very little influence of the lipid nature (Table 1).

3.2. Ketoconazole stability to UV irradiation
In our previous papers [19,20] dextran hydrogels were obtained by UV-induced cross-linking of DEX-MA. Unlikely KTZ is a photosensitive drug [7]. Therefore a set of preliminary experiments were performed in order to assess if drug encapsulation into SLN may protect it against UV induced degradation. To this end, KTZ loaded SLN prepared with the three lipid phases were submitted to irradiation at 310 nm for 4 hours together with solutions of the drug in methanol, used as a control. All the samples were analyzed by HPLC and the results are reported in Figure 1.

It could be observed that KTZ methanolic solutions underwent a progressive degradation and turned brown-colored, with less than 6% of drug recovered after 4 h of UV irradiation. The percentages of residual drug were much higher in the SLN samples, suggesting that the degradation mainly occurred on the particles surface, with a trend dependent on the melting point of the lipid. In fact Compritol, the higher melting lipid, seemed to be able to protect KTZ more efficiently than Precirol, added or not with almond oil. It is likely that the mobility of the drug within SLN was restricted, therefore it moved towards the nanoparticles surface in lesser extent during the irradiation time. However the encapsulation reduced deeply but not completely the drug degradation; therefore, in order to avoid the loss of not negligible amount of active principle, the hydrogels were produced by chemical reagents, according to a procedure reported in the literature [22].

3.3. Hydrogels preparation and release studies
The hydrogels containing the three SLN formulations (SLNPK, NLCPOK, SLNCK) were submitted to release studies. The release profiles are reported in Figure 2.
The lipid nature influences the drug release profile, in fact SLN<sub>PK</sub> release KTZ at a faster rate than SLN<sub>CK</sub> and NLC<sub>POK</sub> even if, in any case, all the loaded ketoconazole is released within 6 hours. No significant differences could be evidenced when dextrans with different derivatization degree were employed (Figure 3).

3.4. Rheological measurements

Rheological behaviour is a crucial characteristic for the application of the hydrogels in topical formulations [31]. A topical dosage form needs to have a semisolid consistency, to be easily spreadable on the skin or able to remain in the application site when employed for vaginal applications. In our previous study, the effect of the lipid phase on the rheological behaviour of dextran hydrogels loaded with different SLN, was investigated [20]. SLN samples showed mechanical spectra, recorded in the range 0.01 – 10 Hz, typical of a viscoelastic liquid with the dissipative modulus G'' bigger than the elastic one G' [16]. After the addition of DEX-MA and photo cross-linking reaction, the mechanical spectra changed deeply and the systems showed weak gel behaviour. In particular, the gel strength was affected by the lipid phase as well as by the derivatization degree of DEX-MA. Taking into account these previous results, in order to obtain formulations with a suitable texture [31,32], DEX-MA<sub>20</sub> was employed for SLN samples.
prepared with lipids having higher melting point, whereas DEX-MA$_{40}$ was used for the other ones. Therefore, DEX-MA$_{40}$ (2.5% w/w) was added to the nano-suspensions of SLN$_P$ and NLC$_{PO}$ and after chemical reticulation the mechanical spectra reported in Figure 4 were obtained.

Figure 4. Rheological studies. Mechanical spectra of the hydrogels SLN$_{PK}$/DEX-MA$_{40}$ (▲ $G'$; △ $G''$) and NLC$_{PO}$/DEX-MA$_{40}$ (■ $G'$; ◀ $G''$) at 25.0 ± 0.2°C.

G' and G'' values were influenced by the lipid nature and for SLN$_P$ resulted comparable with those of the hydrogels obtained by photo-crosslinking [20]. DEX-MA$_{20}$ at 2.5% w/w was employed for SLN$_C$ samples and the relative mechanical spectrum is reported in Figure 5 and compared with the system containing SLN$_P$.

Figure 5. Rheological studies. Mechanical spectra of the hydrogels SLN$_{CK}$/DEX-MA$_{20}$ (♦ $G'$; ◊ $G''$) and SLN$_{PK}$/DEX-MA$_{20}$ (▲ $G'$; △ $G''$) at 25.0 ± 0.2°C.

Figure 6. Rheological studies. Mechanical spectra of the hydrogels SLNPK/DEX-MA$_{20}$ obtained with different amount of the polymer: 5% w/v (♦ $G'$; ◊ $G''$), 3.75% w/v (▲ $G'$; △ $G''$) and 2.5% w/v (■ $G'$; ◀ $G''$) at 25.0 ± 0.2°C.
It is evident that the nature of the lipid phase influences the gel strength. All the systems showed a weak gel behaviour, with the elastic modulus $G'$ bigger than the dissipative modulus $G''$, a $G'/G''$ ratio $<10$, and values of both modulus quite independent from the applied frequency. The influence of the polymer concentration was also evaluated. The mechanical spectra obtained on samples of SLN$_P$ after addition of DEX-MA$_{20}$ at different concentrations and cross-linking reaction are reported in Figure 6.

The consistence of the formulation can be opportunistly modulated varying the concentration of the polymer, because the values of $G'$ and $G''$ are related to DEX-MA concentration and increase with this later. In all the samples the viscosity is dependent from the shear stress; in particular $\eta$ decreases when $\gamma$ increases, according to a behaviour characteristic of pseudo-plastic systems (Figure 7).

3.5. Microbiological studies
We investigated the in vitro antifungal activity of SLN$_{PK}$ DEX-MA hydrogel, free ketoconazole and a topical skin formulation available on the market (Nizoral). For the purpose of this study we have chosen to incubate the samples for different time with an inoculum of $1 \times 10^6$ as previously described with some modifications [26]. As it can be observed in Figure 8, the samples of SLN$_{PK}$ DEX-MA hydrogel induced the growth inhibition of Candida albicans ATCC 24433 with a reduction of cells growth at pH 5 after 4h of incubation, respect to untreated control. A remarkable reduction of cells viability was observed at 48h of incubation at both pH 5 and 7. Finally, no appreciable differences between our preparation and the marketed one were observed.

We also evaluated the effect of the pH of the medium on antifungal activity, because is known the susceptibility of ketoconazole activity to environmental pH [33]. As seen in Figure 8 different pH did not interfere with sample antifungal activity.

3.6. In-vivo studies: primary skin irritation experiments
Primary skin irritation experiments were carried out on rabbits, in order to verify the use of the system SLN/dextran hydrogel as topical formulation. In particular DEX-MA hydrogels containing or not SLN$_P$ and SLN$_{PK}$ were tested according to the procedure described in section 2.12. Individual results of skin irritation scores were always equal to 0.00. In the animals erythema or edema were absent after 24, 48 and 72 h of application of the three samples (DEX-MA hydrogel, SLN$_P$/DEX-MA hydrogel, SLN$_{PK}$/DEX-MA hydrogel) as well as in the control. The PH of all the components was negligible, indicating the absence of skin irritation and, as a
consequence, the possibility to employ the system SLN<sub>PK</sub>/DEX-MA hydrogel in topical formulations.

Figure 8. Microbiological studies. In vitro activity of SLN<sub>PK</sub>/DEX-MA hydrogel, a commercial cream (Nizoral) and a ketoconazole solution against Candida albicans ATCC 24433. Reported results refer to an inoculum of 1x10<sup>6</sup> cells/ml and to a drug concentration of 50 µg/ml. The pH of media was 5.0 and 7.0. Data represent means ± standard deviation from three independent experiments, each performed in triplicate.

CONCLUSION

Ketoconazole can be efficiently encapsulated into solid lipid nanoparticles that are able to deliver the drug in a sustained manner without compromising its activity. The incorporation of these nanoparticles into DEX-MA hydrogels gave rise to a biocompatible system, whose rheological properties can be conveniently and easily adjusted changing the derivatization degree and/or the concentration of the polymer, in order to assure a semisolid consistency, a good spread-ability and a pleasant texture, or to remain in the application site when necessary. Overall these results suggest a good potential of the system SLN/dextran hydrogel for the topical modified delivery of ketoconazole.

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