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Lipid-based nanocarriers for Rose Bengal dermal delivery: a promising approach in melanoma treatment

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Abstract: Rose Bengal (RB) is a photosensitizer used to eradicate cancer cells. 10% RB demonstrated to kill melanoma but, nowadays, RB biopharmaceutical profile limits its clinical applications. This work focuses on the development of RB lipid nanocarriers, solid lipid nanoparticles (SLN) and transfersomes (TF), to treat melanoma via dermal delivery in the absence of light. Preformulative studies identified the best manufacturing and formulative parameters and leader formulations were obtained. Particle size of SLN and TF were around 130 nm and 200 nm respectively, both carriers were homogenous and stable in 30 days. UV-spectrophotometer and fluorometer analysis showed RB maximum absorption and emission wavelength red-shifted demonstrating an association of dye with the lipid phase. Ex-vivo permeation studies showed that RB solution did not permeate through stratum corneum (SC) while TF increased RB delivery up to 66%, on the other side SLN were found to be retained in the SC. We obtained two stable RB lipid nanocarriers potentially active in eradicating melanoma via dermal delivery.

Keywords: Rose Bengal; melanoma; solid lipid nanoparticles; transfersomes; dermal delivery.

1. Introduction

RB is a dye used in ophthalmic diagnostic [1]. Despite this, RB is a photosensitizer employed in photodynamic therapy, and it demonstrated intrinsic cytotoxicity against tumor and microbial cells [2–4]. PV-10® is a 10% RB solution intralesional injected currently tested in cancer treatment, in particular melanoma towards which RB showed high cytotoxicity [4]. Nevertheless, RB biopharmaceutical profile limits its clinical application: RB is amphiphilic with hydrophilic tendency limiting its accumulation in cells, and it has a short half-life requiring multiple administration to reach the target site if administered systemically [5,6]. To date, different RB delivery systems were developed proven to overcome the aforementioned limits [7]. Lipid-based nanosystems, such as SLN and TF, were efficiently employed for dermal delivery of drugs. SLN are nanoparticles (NP) characterized by a solid lipid matrix able to form an occlusive film on the skin surface, in this way they reduce transepidermal water loss improving drug penetration and controlling its release. TF consist of a hydrophilic core surrounded by a hydrophobic bilayer; the presence of surfactant makes TF ultradeformable since they can squeeze and pass through skin pores smaller than their size releasing drug in the deepest region of the body[8]. Considering this background, we aim to develop and characterize RB loaded SLN and TF to treat melanoma via dermal delivery in the absence of light. We obtained two potential nanocarriers for RB topical delivery.

2. Experiments

2.1. Materials

Rose Bengal sodium salt (RB), cholesterol, Span® 80, dichloromethane and ethanol were purchased by Sigma-Aldrich (St. Louis, MO). Witepsol® E85 was supplied by Cremer Oleo (Hamburg, Germany); Gelucire® 44/14 was gifted from Gattefossé SAS (Saint-Priest, France). Benzalkonium chloride (BAC) was acquired by Cruciani Prodotti Crual (Rome, Italy). Lipoid S 100 was gifted by Lipoid GmbH (Ludwigshafen, Germany). Phosphate-buffered saline (PBS, NaCl 0.138 M; KCl 0.0027 M; pH 7.4; 25 °C) were obtained by Sigma-Aldrich (Milan, Italy). Ultrapure bi-distilled water was obtained by a MilliQ R4 system, Millipore (Milan, Italy).

2.2. Preparation of SLN

SLN were prepared by modified W/O/W double-emulsion technique [9]. A preformulative study tested BAC concentrations and sonication times (Table 1). SLN were prepared dissolving Witepsol® E85 (111.11 mg) and Gelucire® 44/14 (88.89 mg) in 5 mL of dichloromethane, 0.5 mL of MilliQ water were added and homogenized by probe sonicator Bioblock Vibracell (Fisher Bioblock Scientific, Illkirch, France) for 30s at 70% US. This primary emulsion was added to 10 mL BAC aqueous solution and sonicated testing different times (Table 1). This double emulsion was placed for 3 h under magnetic stirring at room temperature (r.t.) to remove the organic solvent and obtain SLN. Leader blank SLN (SLNb) and RB SLN (SLN-RB200, SLN-RB500) were prepared as above considering the outcomes of preformulative study, for loaded SLN RB aqueous solution (0.004 M for SLN-RB200; 0.01 M for SLN-RB500) was used instead of MilliQ water.

Table 1. Formulative parameters modified during preliminary studies on SLNb.

Formulation	BAC (%w/v)	Sonication time (s)
A	1.0	20
B	1.0	30
C	1.0	90
D	1.0	120
E	0.5	90
F	1.5	90

2.3 Preparation of TF

Two series of TF were developed, TF1 and TF2. About TF1, TF1-A and TF1-B were studied (Table 2). TF1-A were prepared by thin-film hydration method (TFH), TF1-B by reverse-phase evaporation method (REV) [10]. To obtain TF1-A 100.8 mg of Lipoid S 100, 31.29 mg of cholesterol and 16 μL of Span® 80 were dissolved in a mixture of ethanol, methanol and chloroform (2:2:1) which was completely evaporated under vacuum (u.v.) at 40 °C. The lipid film formed was hydrated with 20 mL of MilliQ water for 1 hour at 40°C by a rotary evaporator (Rotavapor RE111, Büchi LabortechnikAG, Flawil, Switzerland). The dispersion was kept for 1 hour at r.t. and sonicated testing two techniques (Table 2). TF1-B were prepared dissolving 100 mg of Lipoid S 100, 20 mg of cholesterol and 10 μL of Span® 80 in a mixture of diethyl ether and chloroform (3:1), 10 mL of MilliQ water were added and sonicated for 4 min at 50% US providing an emulsion which was evaporated u.v. at 40 °C until complete solvent evaporation. The gel formed was kept at r.t. overnight to be converted into the transfersomal dispersion which was sonicated testing two sonications (Table 2). Blank TF1 (bTF1-A, bTF1-B) and loaded TF1 (RBTF1-A, RBTF1-B) were prepared considering the outcomes of the preformulative study, loaded TF1 were obtained using 2 μM RB aqueous solution instead of MilliQ water. TF1 were stored for 3 months at 4°C to identify the best preparative technique. Loaded TF2 (RBTF2) were prepared based on TF1 study changing solvent and sonication time (Table 2). 200-

RBTF2 and 500-RBTF2 were prepared by REV technique: 400 mg of Lipoid S 100, 75 mg of cholesterol and 40 μL of Span® 80 were dissolved in ethanol at 50 °C and mixed with 10 mL of RB aqueous solution (200 μM for 200-RBTF2; 500 μM for 500-RBTF2), sonicated for 30s at 50% US amplitude and evaporated u.v. at 50 °C until complete solvent evaporation. The dispersion was kept for 1 hour at r.t. and sonicated by 3 sonication cycles: each cycle consisted of 10 s of US followed by an interval of 20s [11]. TF2 were extruded 5 times through a 0.45 μm polycarbonate membrane and 5 times through a 0.2 μm polyethersulfone membrane. Corresponding blank (bTF2) was prepared.

Table 2. Formulative parameters modified during preliminary studies on TFs.

Formulation	Technique	Solvents	Sonication
TF1-A	TFH	EtOH MeOH CHCl3	Probe 50% (60s,90s, 120s) Bath 40°C (5′, 10′, 15′)
TF1-B	REV	C4H10O CHCl3	Probe 50% (60s,90s, 120s) Bath 40°C (5′, 10′, 15′)
TF2	REV	EtOH	45% US

2.4. Analysis of particle size, polydispersity and zeta potential

Particle size and polydispersity index (PDI) were determined by photon correlation spectroscopy using a Coulter nanosizer N5 (Beckman-Coulter Inc., Miami, FL, USA). Zeta-potential was determined by Zetasizernano using the M3-PA LS (Phase Analysis Light Scattering) technique.

2.5. Evaluation of interaction between RB and lipid phase

An evaluation of the interaction between RB and lipid phase was performed. SLN-RB200; SLN-RB500; 200-RBTF2; 500-RBTF2 were diluted with MilliQ water to obtain 8 μM RB concentration, the absorption and emission spectra were recorded by UV-Spectrophotometer (SHIMADZU UV-1800, Kyoto, Japan) and RF-6000 spectrofluorometer (Shimadzu, Kyoto, Japan) respectively. Results obtained were compared to corresponding values of 8 μM RB aqueous solution.

2.6. Physical stability studies

SLN were stored both at 25 °C and 4 °C and diameter and PDI were analyzed during the time (1, 7, 15, 30 days). TF2 were stored at 4°C and analyzed as above. At the time of writing this manuscript, the stability of 200-RBTF2 was recorded until 15 days.

2.7. Ex-vivo permeation study on stratum corneum (SC)

Ex-vivo permeation of SLN-RB500 and 500-RBTF2 was evaluated on excised pig SC using a 500 μM RB aqueous solution as control. Porcine skin was obtained by a local slaughterhouse and properly prepared[12]. The experiment was performed using a 12-multi-well cell culture plates suitably modified (project INCREASE SARDINIA 2016-17, protocol number 31351, University of Sassari). The wells were filled with PBS and SC was put on the plates with the internal side in contact with PBS. 300 μL of samples (0.15 mg of RB) were put on top of SC, the plates were placed into the incubator shaker SKI 4 Shaker Incubator (ARGO LAB, Carpi, Italy) at 70 rpm and 32 °C. At each time point (30 min, 1h, 3h, 6h, 24h) samples (200 μL) were withdrawn from the wells and replaced with an equal volume of fresh medium. 1 mL of dimethyl sulfoxide (DMSO) was added to each sample to extract RB, the amount of RB permeated was measured by UV-spectrophotometer and calculated referring to the calibration curve prepared in DMSO. To evaluate RB retention in SC, tissue samples were

boiled with 2 mL PBS for 10 min, then 2 mL of DMSO were added to extract RB and further boiled for 10 min. The amount of RB retained in SC was calculated by the same method employed above.

3. Results

3.1. Preparation of SLN

Table 3 shows results of SLN preformulative study. 20s of sonication was unsuitable due to rapid phase separation, 30s provides a homogeneous dispersion and particle size was 275.6 (PI=0.313). Prolonging sonication to 90s a decrease in SLN size and PDI was observed but a further increase led to the formation of visible aggregates. The best BAC concentration was 1% (w/v): 0.5 % led emulsion breaking and phase separation, on the other side 1.5 % allowed to obtain a homogeneous dispersion and acceptable size but, after one day, SLN aggregation and dispersion gelification were observed. Formulative parameters selected for SLN were 1% (w/v) BAC and sonication of 90s.

Table 3. Dimensional properties (particle size and polydispersity index, PDI) and visual observation of formulation prepared during preliminary studies

Formulation	Particle Size (nm±SD)	PDI (±SD)	Visual observation
A	-	-	Phase separation
B	275.6±35.4	0.313±0.036	Homogeneous dispersion
C	138.7±3.0	0.263±0.032	Homogeneous dispersion
D	> 1000	> 0.4	Aggregation
E	265.9±37.1	0.136±0.029	Phase separation
F	145.0±48.6	0.137±0.021	Aggregation and gelification

3.2. Preparation of TF

Figure 1 shows the effects of TF1 sonication. Not sonicated TF1-A had a size of 659.03±10.54 nm, TF1-B size was above 1µm. 2 min of probe sonication at 50% US amplitude was selected as sonication method since it allowed to obtain vesicles of 182.47 nm for TF1-A and 206.63 nm for TF1-B. Bath sonication (Bandelin Sonorex RK 52 Heinrichstrable, Berlin, Germany) of TF1-A revealed a good dimensional profile but it was discarded due to the presence of dispersed aggregates. RBTF1-A, RBTF1-B and corresponding blanks were prepared using selected sonication parameters. Storage stability showed that TF1-B was the most stable: RB TF1-A size increased from 214.4±4.38 to over 1 µm, RB TF1-B increased from 297.6±1.41 nm to 437.65±4 nm; a similar increase was seen for bTF1. Considering these outcomes, TF2 were prepared by REV technique and probe sonication method.

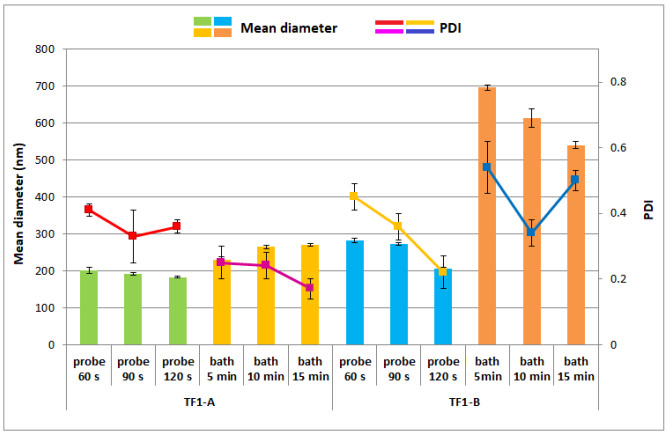


Figure 1. Influence of sonication method and sonication time on blank TF1 formulation dimensional properties (particle size and polydispersity index, PDI)

3.3. *Analysis of particle size, polydispersity and zeta potential*

The dimensional profile and zeta potential of SLN and TF2 are shown in table 4.

Table 4. Particle size, PDI and zeta potential of SLN and TF2

Formulation	Particle Size (nm±SD)	PDI (±SD)	Zeta potential
SLNb	130.1±3.01	0.225±0.02	61.2±2.4
SLNRB-200	133.0±3.19	0.231±0.01	64.5±1.1
SLNRB-500	135.4±03.35	0.220±0.01	63.5±1.5
TF2b	219.11±1.79	0.23±0.09	-4.9±0.9
200-RBTF2	202.77±2.06	0.28±0.01	-26.0±0.2
500-RBTF2	230.67±1.02	0.20±0.02	-48.7±0.4

3.4. *Evaluation of interaction between RB and lipid phase*

Table 5 reports maximum absorption and emission wavelength (λ_{max}) values of RB in water compared to RB Formulated in lipid nanocarriers.

Table 5. Absorption and emission values of RB in water and RB formulated in nanocarriers.

	Abs (nm)	Ems (nm)
RB	549	568
SLN-RB200	565	581
SLN-RB500	564.5	582
200-RBTF2	562	582
500-RBTF2	562.5	583

3.5. *Physical stability studies*

Particle size and PDI of SLN did non significantly change when NP were stored at r.t. SLN aggregated at 4°C, indeed size and PDI increased in one week for all formulations. since TF2 components need to be stored at 4°C, the r.t. storing was non studied. Particle size and PDI of TF2 did not change significantly, it was only observed a slight decrease of PDI values (Figure 4).

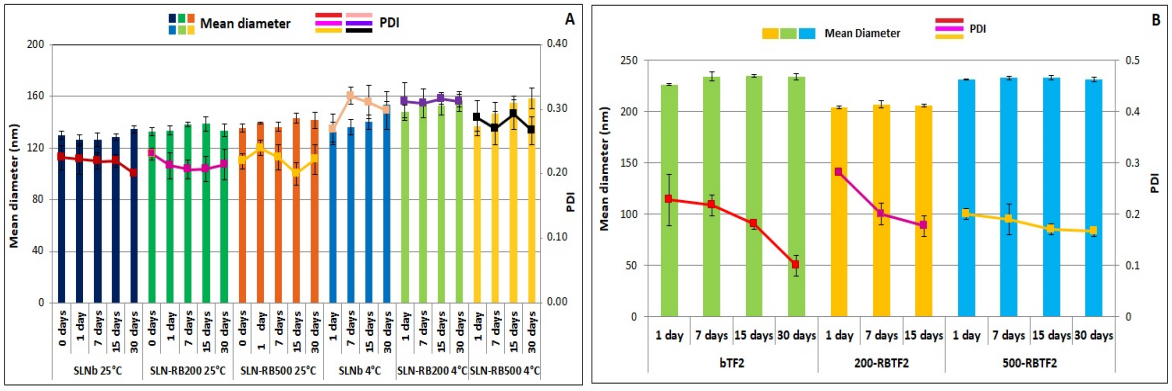
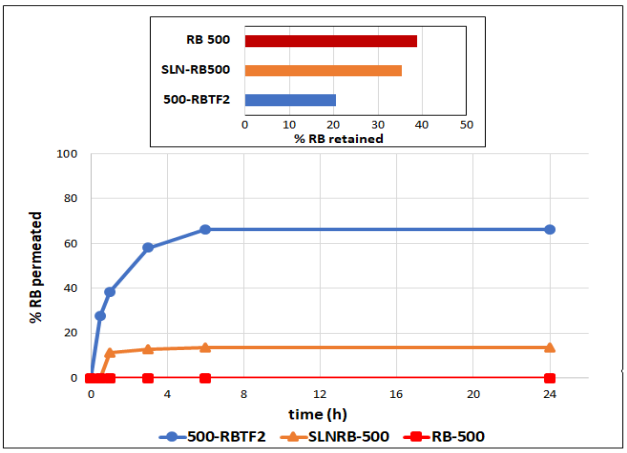


Figure 2. Physical stability study of **(a)** SLN and **(b)** TF2

3.6 *Ex-vivo permeation study on stratum corneum (SC)*

Figure 3 reports the cumulative amount of RB permeated over 24h and the amount of RB retained in SC after 24h. Permeation profile showed that, after 24h, RB aqueous solution did not permeate through SC whereas formulating RB in SLN or TF improved its permeation by 13.7% and 66%

161 respectively. On the other side, RB amount retained in SC was the highest for free RB (38.82%),
162 followed by SLN-RB500 (35.26%) and 500-RBTF2 (20.50%).



163

164 **Figure 3**. Cumulative amount of RB permeated through SC and retained in SC from RB aqueous solution,
165 SLNRB-500 and 500-RBTF2.

166 **4. Discussions**

167 RB demonstrated to be active in killing melanoma cells but its clinical application is limited by
168 its biopharmaceutical profile. We formulated RB in SLN and TF to improve its efficiency in treating
169 melanoma. To obtain TF we performed a two-step preformulative study: TF1 allowed us to identify
170 the best manufacturing parameters (preparative technique and sonication method) and formulative
171 parameters (sonication time and organic solvent) were refined by TF2. Size of TF prepared by REV
172 technique (TF1-B) was the highest, this can be explained considering that REV incorporates a higher
173 amount of water, indeed it is considered the most suitable technique to encapsulate water-soluble
174 drugs[13]. Moreover, the storage stability revealed that TF1-B was the most stable formulation. TF2
175 were prepared by REV method employing ethanol as solvent to obtain a safe formulation as it is a
176 solvent with low toxic potential, according to Pharmacopoea Italica XII edition. Sonication of
177 formulations improves NP dimensional profile. Probe sonication allowed to obtain the lowest particle
178 size and PDI values, as reported by Hadian *et al* that evaluated the effect of sonication on similar lipid
179 vesicles[14]. Since TF components are heat-sensitive, TF2 were sonicated in cycles to allows TF to cool
180 and avoid thermal degradation[11]. Similar behavior was found for SLN: particle size and PDI
181 decreased as the sonication time increased but, when sonication exceeds 90s, aggregation phenomena
182 were observed. Surfactants are a key component to provide physical stability to colloidal systems;
183 during SLN preformulative study PVA, tween®80 and BAC were tested. The incorporation of PVA
184 and Tween® 80 did not lead to any stable formulation (data not shown), but BAC has proven useful
185 for this purpose. BAC is a solubilizing and wetting agent widely use as antimicrobial preservative in
186 pharmaceutical formulation and cosmetics, moreover it has enhanced the topical penetration of
187 lorazepam[15]. Here in BAC is employed as a cationic surfactant to stabilize SLN, as previously
188 reported[16]. 1% w/v BAC concentration revealed to be the ideal one for our system. The dimensional
189 analysis revealed nanocarriers were below 250 nm and in a state of monodispersity distribution.
190 Similar values of zeta potential were found for loaded and unloaded SLN revealing that RB did not
191 influence SLN surface charge; on the other side, zeta potential of TF became progressively more
192 negative as RB loaded concentration increased. NP size and surface charge are fundamental for both
193 storage stability and skin penetration. To obtain stable formulations, zeta potential should be below
194 30 mV or above 60 mV as repulsion of charged NP prevents them from aggregating[14]. It can be
195 assumed that SLN and TF2 were stable, as emerged from stability studies. Lipid vesicles whose size
196 was between 100-600 nm proved to enhance skin penetration of water-soluble photosensitizer drugs.
197 SLN around 100-200 nm showed the same results[8]. The study of Hugo *et al* revealed RB interacts

with liposomal vesicles as RB absorption λ_{max} red-shifted when it is associated with liposomal membrane compared to water[17] and our analysis showed similar results; we can suppose that RB bounded to lipid components of SLN and TF. The preliminary permeation study revealed free RB did not permeate according to its chemical profile: it is a water-soluble drug with high molecular weight (PM=1017.64 g/mol) limiting its penetration across the skin. Due to RB amphiphilic character, a part of it was retained by SC. NP showed different behavior. TF proved to increase RB permeation as they can squeeze along the intracellular lipid of SC. SLN slightly increased RB permeation but most of them were tissue-retained due to their affinity for lipid skin. In this way, they prolong RB interaction with SC and, at the same time, enhance its penetration into the deepest layers[8].

5. Conclusions

We formulated RB loaded TF and SLN to determine if they can improve RB delivery to melanoma. NP were in a dimensional range suitable for topical delivery and they proved to interact with RB. The preliminary permeation study reported that TF permeated through SC. As melanocytes reside in the basal epidermis and on the top of the dermis [18], TF could be employed to reach melanoma cells. SLN were mainly found within SC so they could be considered to treat skin disease involving SC itself, with the advantage of protecting RB from undesirable light activation[8]. Cytotoxicity studies on melanoma cells are ongoing, and further physical-chemical characterizations are planned.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

RB: Rose Bengal
SLN: solid lipid nanoparticles
TF: transfersomes
SC: stratum corneum
TFH: thin film hydration
REV: reverse phase evaporation
NP: nanoparticles
BAC: benzalkonium chloride
r.t: room temperature
u.v: under vacuum

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