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***Advanced Rose Bengal Delivery Systems for the local and
systemic management of cancer.***

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Outline

The PhD thesis “Advanced Rose Bengal Delivery Systems for the local and systemic management of cancer” intends to address some aspects of cancer management that have not yet been fully accomplished. This PhD thesis proposed exploiting the biological properties of Rose Bengal, a hydrosoluble compound with recognised pharmacological effects in various diseases through advanced delivery systems. The first chapter, focused on Rose Bengal (RB), offers a comprehensive introduction to the investigated molecule from a biomedical point of view, providing the benefits, limitations, and challenges posed by its formulation and delivery. In this regard, the panorama of RB’s delivery systems already developed and reported in the literature is detailed and discussed. The second chapter represents the central part of the Thesis, and it is devoted to managing local and systemic melanoma. A nanosized transferosome dispersion was projected, developed, and characterised for RB encapsulation and selective delivery to melanoma cells. Mainly, this chapter comprises three published manuscripts and a further unpublished section which go through the transfersome dispersion development and its conversion into semisolid and solid dosage forms (polymeric and hollow microneedles, respectively) to provide a dosage form finally. Lastly, the third chapter briefly introduces a different topic: the fabrication of a 3D-printed implantable device loaded with RB polymer-based formulation for local cancer therapy.

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Chapter I.

Introduction

This section of the thesis has been published as a Review Article in the peer-reviewed and indexed Journal “*Dyes and Pigments*”; (2021 Journal Impact Factor (JIF): 5.12; JIF Quartile: Q1 in the categories Applied Chemistry and Material Science, Textiles, Q2 in the category Chemical Engineering). The Review Article was the result of the first academic year of the Ph.D. (2019-2020) when laboratory activities were forbidden due to the COVID-19 Pandemic. This Review Article is dedicated to all the Scientists that have spent these last years studying the COVID-19 virus, thus developing the vaccine that helped fight the pandemic.

Review Article

Nanotechnology-Based Rose Bengal: A Broad-Spectrum Biomedical Tool

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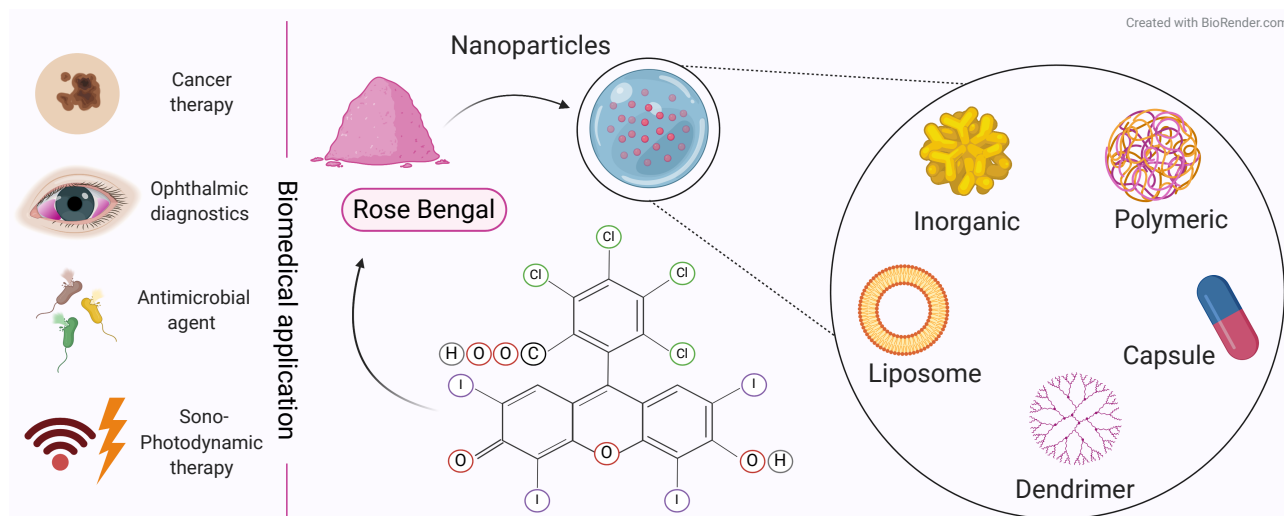
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Abstract

Rose Bengal is an anionic xanthene dye, a derivative of fluorescein, born in the 19th century as a textile dye; to date, it is known primarily in ophthalmology as a diagnostic tool and demonstrated to be promising in the biomedical field. Rose Bengal intrinsic cytotoxicity against tumor and microbial cells is responsible for its therapeutic potential; moreover, it is a sono-photosensitizer drug suitable for sono-photodynamic therapy. Due to its disadvantageous bio-pharmaceutical profile, Rose Bengal is approved only as an ocular diagnostic stain and has been designated by the Food and Drug Administration as an orphan drug to treat certain cancers. Two main pharmaceutical approaches such as molecular structural changes and drug delivery systems were investigated to overcome Rose Bengal limits. The current review reports the physical-chemical profile and pharmacokinetic properties of Rose Bengal and presents a short overview of its biomedical applications. The review focuses on Rose Bengal nanosized delivery systems already developed to outline the current research topics and to assess whether nanotechnology can maximize its therapeutic potential overcoming its limits. Through the revision of Rose Bengal research articles about this topic, it emerges that a proper Rose Bengal nanosized delivery system can efficiently improve its biopharmaceutical profile and enhance Rose Bengal-based therapy.

Keywords: *Rose Bengal, Nanotechnology, Drug Delivery, Nanoparticles, Photosensitizers, Photodynamic therapy.*



1. Introduction.

Rose Bengal (RB) (Color index name Acid Red 94) is a purplish dye discovered in the 19th century when Ghnem added halogens to fluoresceine to synthesize new dyes for wool (German patent n. 32584). The name is a name of fantasy and is probably due to its similarity to the pink dot drawn in the center of the forehead of married women of Bengali, a region of India [1,2]. Chemically, RB is a highly water-soluble dianionic fluorescent dye that belongs to the class of xanthene dyes; the molecule initially synthesized consisted of two iodine atoms, but it was subsequently modified by adding two further iodine atoms to obtain the current form of RB, the 3', 4', 5', 6'-tetrachloro-2,4,5,7-tetraiodo-fluorescein [2]. RB is cheap, biocompatible, widely available, and primarily used as a colorant, especially in Japan, where it has also been approved as a food colorant in 1973 [3]. Its coloring abilities of bacterial cells and protozoa or tissues are significant for this review; however, there are conflicting opinions in the bibliography regarding if considering or not RB as a vital dye [1,4,5]. Besides, RB exhibits intrinsic toxicity properties towards different cancer [6–9] and microbial [10–13] cell lines, as well as sono-photochemical properties which allow using the dye in photodynamic (PDT) and sonodynamic therapy (SDT) in the context of tumor and bacteria eradication and the dermatological/cosmetic field [14–17].

Despite its demonstrated properties and therapeutic potential, RB is currently approved only as an ocular diagnostic instrument [4,5] and has been designated by the Food and Drug Administration (FDA) as an orphan drug to treat certain cancers. The limited use of RB as a therapeutic agent relies mainly on its hydrophilic tendency [18,19] and reduced half-life [20] making RB hard to cross cell membranes negatively impacting cell uptake, cell accumulation, and biodistribution. Moreover, the half-life of 30 minutes is a further barrier to proper tissue accumulation, and multiple administration would be required to reach the therapeutic window [16,18,21]. Two different pharmaceutical approaches have been investigated to overcome RB limits and maximize its therapeutic potential: molecular structural changes and technological development of drug delivery systems (DDS). In both cases, the final result needs to provide biocompatibility, RB distribution only to target cells and should not negatively influence its sono-photochemical properties [22,23].

Herein, we discuss the physical-chemical properties of RB and the pharmacokinetic aspects relevant to its application as therapeutic agents. We outline RB medical development since its discovery until nowadays to give a comprehensive historical outlook of this emerging drug. Further, we describe RB delivery systems already studied briefly comparing the technological approach to the molecular structural changes approach. The review aims to determine whether drug delivery

science has allowed exceeding RB limits and what aspects it has improved. In the last decades, interest in RB has progressively increased (Figure 1), but the number of review articles about the topic is insufficient. Review articles yet present in literature analyze RB photochemistry and photophysics and its role in organic synthesis, biomedical application of sono-photoactive RB, and RB as a dye for ophthalmic diagnostic purposes and in melanoma treatment. As an increasing number of RB nano-based delivery systems are under investigation, a comprehensive overview of the findings obtained to date is necessary. To the best of our knowledge, this is the first review about RB nano-based delivery systems.

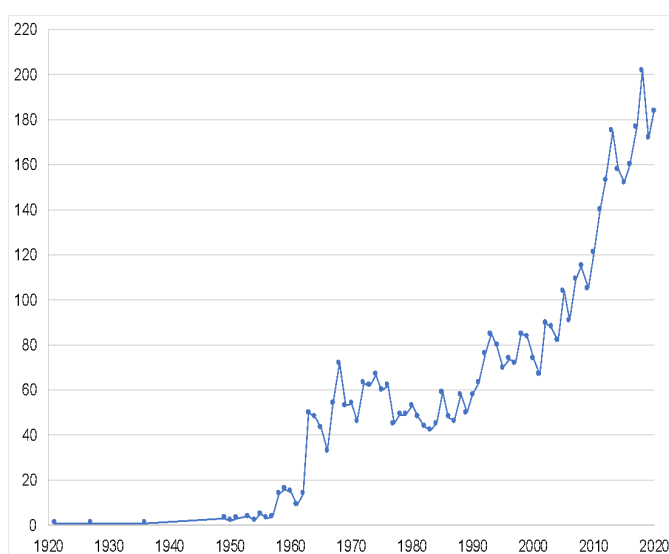


Fig. 1. PubMed timeline RB results by year. (pubmed.ncbi.nlm.nih.gov/?term=rose%20bengal updated to 31/12/2020).

2. Physical-chemical profile and pharmacokinetic properties.

RB is an organic compound consisting of a xanthene ring substituted in C9 position by a tetrachloro benzoic acid, these two moieties have an almost orthogonal orientation which makes them approximately independent (Figure 2). RB parent molecule can be salified in C3 hydroxyl and C6 carboxyl to obtain different salts; actually, the most commonly used form of RB is its disodium salt RBB (RBB disodium salt). Table 1 reports some of its physical-chemical properties.

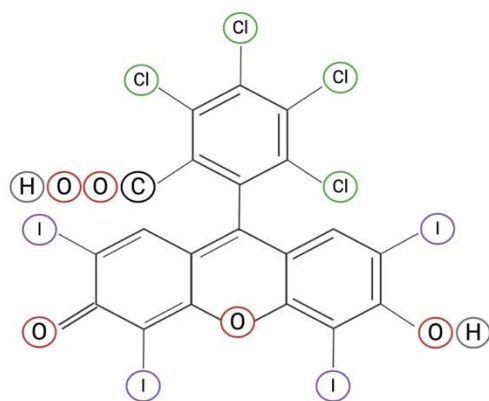


Fig. 2. RB chemical structure.

Table 1. RB and RBB physical-chemical profile

	<i>Rose Bengal</i>	<i>Rose Bengal Disodium Salt</i>
<i>Molecular weight</i>	973.7 g/mol	1017.6 g/mol
<i>Hydrogen bond donor count</i>	2	0
<i>Hydrogen bond acceptor count</i>	5	5
<i>Physiological charge</i>	-2	-2
<i>log P (log partition coefficient octanol / PBS)</i>	0.66	0.59
<i>Water solubility</i>	n.f.	100 mg/ml
<i>Ethanol solubility</i>	n.f.	30 mg/ml

n.f.= not found

The halogenated xanthene ring in the structure is the main responsible for its photochemical properties: RB is a photosensitizer (PS) drug, a molecule that, after absorbing photons, can alter another molecule in the system by the generation of reactive oxygen species (ROS) [15,24–26]. Rauf et al [27] have determined the maximum wavelength at which RB absorbs and emits in different solvents, reporting that the maximum absorption in water is 546 nm and the emission at 567 nm. In the case of RB, the addition of halogens with a higher atomic number (heavy atoms effect)

increases the singlet oxygen (1O_2) quantum yield directly influencing the rate of intersystem crossing, crucial for generating RB triplet excited state ($^3RB2^*$) from the singlet excited state ($^1RB2^*$) upon irradiation by visible green light (RB $\lambda_{max}=546$ in water). $^3RB2^*$ subsequently transfers its energy to oxygen in the ground state (3O_2) leading to the ROS 1O_2 with a 1O_2 quantum yield $\Phi=0.76$ [18,28,29].

RB is highly water-soluble, and because of this it can be quickly delivered intravenously. Wachter et al [30] reported that topic hydrophilic formulations had delivered RB to the epidermis rapidly, selectively, and uniformly [30,31]. Moreover, being water-soluble makes it less likely to aggregate in aqueous medium under physiological conditions, a characteristic of hydrophobic drugs: the aggregates can decrease ROS production upon irradiation and consequently the efficacy of a photodynamic treatment [32]. Nevertheless, even RB has demonstrated to form aggregates in solution at a concentration above 2.0×10^{-6} M, affecting its excitation and emission spectra [33].

Buck et al [18] and Pellosi et al [19] have investigated the hydrophilic character of RB and RBB through the determination of the partition coefficient between 1-octanol and water phase. About RB, the logarithm of the partition coefficient ($\log P$) resulted in being 0.66, while for RBB resulted in being 0.59. Considering that lipophilic compounds are characterized by a $\log P > 1.5$ and hydrophilic compounds by a $\log P < 0$, both RB and its salt appeared to be amphiphilic with a more hydrophilic tendency because of the presence of two negative charges at physiological pH, an aspect that remarkably influences its use in medical therapies. The lipophilic character of a drug is essential for its biodistribution and cellular localization, and it results decisive for therapeutic efficiency. As cell membranes are lipophilic, a drug needs to be lipophilic to cross them spontaneously and relocate in intracellular organelles: the poor lipophilicity makes RB hard to cross the cell membrane and the intracellular uptake is weak, resulting in an insufficient cell accumulation at low concentration in the absence of carriers. The two negative charges represent the further limit for the accumulation inside organelles such as mitochondria which have a negative charge potential and are fundamental for programmed cell death mechanisms [16,18,21].

Klaassen investigated RB pharmacokinetics in rats, rabbits, guinea pigs, and dogs reporting a biological half-life of 30 minutes in rats and rabbits, 17 minutes in guinea pigs, and 46 minutes in dogs in an administration range of 0.01-10 mg/Kg. The study also revealed that RB undergoes biliary excretion without being biotransformed [20]. Since half-life determines drug effect duration and influences its body accumulation and distribution, a drug with a half-life of 30 minutes would require multiple administrations to accumulate in target tissue if administered systemically. Based on this,

some RB formulations currently involved in clinical trials are injected directly into the tumor tissue. (ClinicalTrials.gov Identifier: NCT00521053; NCT00237354; NCT02693067; NCT00219843).

3. RB medical application.

3.1 Ocular diagnostic agent.

According to the literature, the first use of RB dates back to 1914 when Römer reported a solution of RB, safranin, and yellow victoria as an ocular antiseptic. In 1919, Kleefeld used Römer's same solution to color, and therefore diagnose, corneal ulcers while Sjögren finally tested a solution of the only RB on healthy subjects in 1933, noting that with a 1% solution no painful lesions or perceptions were found, while a 2% solution caused eye injuries and pain [1,34]. Sjögren subsequently used and recommended a 1% RB solution for keratoconjunctivitis sicca diagnosis; keratoconjunctivitis is confirmed when, after the instillation of the solution, red dots appear in the conjunctiva and cornea, coinciding with the points where the epithelium is desquamated [4]. So, what Rose Bengal is able to color? The initial theory that RB stained the nucleus of dead cells has been largely overcome [4,5]. It has been accepted that RB stains epithelial cells poorly protected by the precocular tear film; in particular, it seems that mucins play a pivotal role in RB diffusion. Mucins are the major macromolecular component of the protected biofilm on the surface of the epithelial cells. The paper by Argüeso and collaborators reports that RB does not stain corneal cells producing mucins since the dye is blocked by binding to mucins on the surface; therefore, the loss of mucin network in patients with dry eyes leads to RB staining [35]. Accordingly, RB is currently approved as an ocular diagnostic agent (Figure 3) and is available at 1% solution or as impregnated sterile strips (Rose Bengal GloStrips™, Bio Rose, Rose Bengal). RB is especially useful to evaluate tear distribution and epithelial integrity of the ocular surface, and it helps in the diagnosis of precocular tear film disorders, mucin precocular deficiency, and corneal abrasions [36].

Parallel to the study of RB in the ocular field, Delprat identifies it as a marker for liver functions. In an article published in 1924 by Delprat et al [37], it emerges that the rate of elimination of the RB from systemic circulation is reduced in the presence of a certain degree of liver dysfunction. In this context, Taplin tested in 1950 the more sensitive radioactive (¹¹³¹-tagged) RB which to date has been replaced by MRI (Magnetic Resonance Imaging) and CT (Computer Tomography) [38].

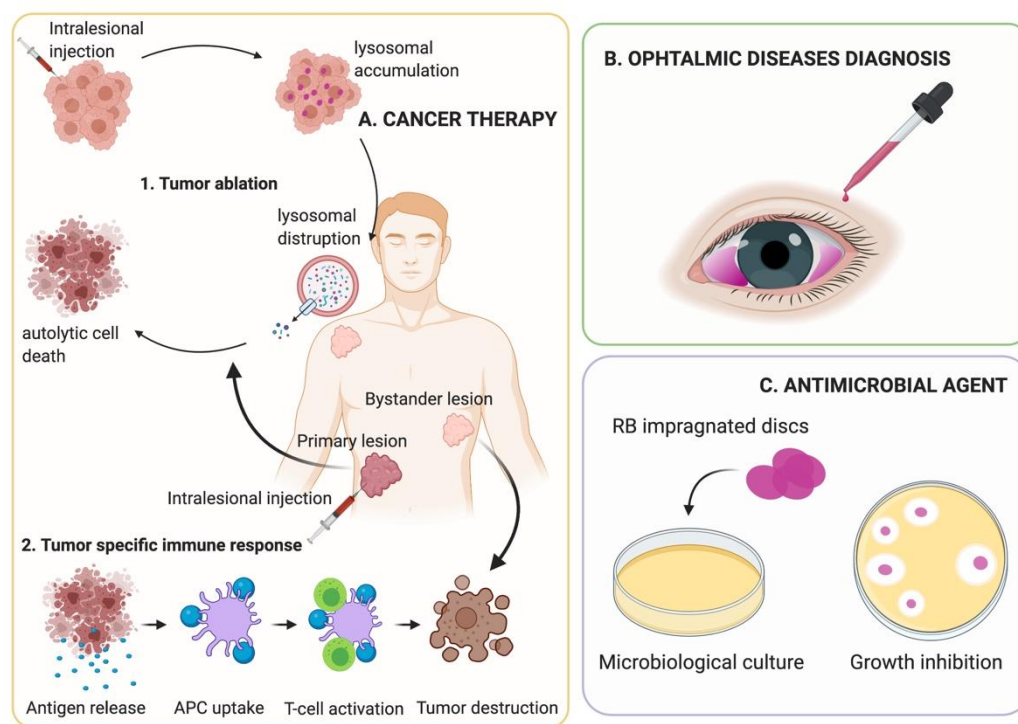


Fig. 3: RB medical application. A. Cancer therapy. RB is injected into the primary tumor lesion causing (1) direct tumor cell death via tumor ablation and (2) indirect tumor cell death following a tumor-specific immune response. B. Ophthalmic diseases diagnosis. RB is blocked from the intact corneal surface by binding to mucins on the surface detecting ophthalmic diseases. C. Antimicrobial Agent. Created with BioRender.com

3.2 Cancer therapy.

The study of RB as an anticancer agent has its roots around 1980, when the Food and Drug Administration (FDA) and the Japanese Ministry of Health, Labor and Welfare, decided to evaluate some food's carcinogenicity additives [3]. In 1986, Ito et al [3] analyzed the induction of thyroid tumors in mice by the oral administration of RB. Mice selected for the study were known to develop tumors spontaneously; they have been divided into three groups: I-a was given 0.5% and I-b 0.125% of RB in water, while I-c was the control group. The administration of RB induced colloid goiters in mice in a dose-dependent way because of the interaction of iodine of RB with the thyroid. On the other hand, from this study, an additional finding emerged: at week 82, the survival rate of the I-a and I-b groups was 94% and 92% respectively, while the survival rate of the control group was 52%. Although the authors did not comment on this result, it represented the starting point for the study of RB as an anticancer drug. According to the literature, low RB concentrations (5-10 μM) can induce cancer cell death after exposure to visible light, but the same RB concentrations cannot induce any relevant damage in the dark [39]. Mousavi et al [7,39] reported RB-induced toxicity in breast cancer and melanoma cell lines: toxicity was evident at 100 μM in both cases and was pronounced at 200

μM for melanoma cells and $300\ \mu\text{M}$ for breast cancer cells. The induction of breast cancer cell death was mainly dependent on apoptosis, both the receptor-mediated pathway and the mitochondrial-mediated pathway were observed. On the other hand, It was initially observed that RB could induce both apoptotic and non-apoptotic cell death in melanoma cell lines, further studies by the same authors clarified that caspase-dependent and -independent pathways were involved in RB-induced apoptosis [40]. ROS generation did not appear to be involved. Similarly, Koevary [8] determined the selective toxicity of RB on ovarian carcinoma cells. In cells cultured with RB for four days, RB-induced growth suppression was evident at $50\ \mu\text{M}$ and maximum at $400\ \mu\text{M}$ and $800\ \mu\text{M}$; RB likewise suppressed the growth when the cells were incubated for only one hour revealing evident toxicity at $800\ \mu\text{M}$. Furthermore, cell damage was provided based on the observation of RB enhanced apoptosis and ROS accumulation in ovarian cancer cells. Anticancer activity of RB was also tested on gastric and colon cancer cells exhibiting the maximum growth inhibition at $300\ \mu\text{M}$ [9,41]. All the studies mentioned above agree that the direct cytotoxicity of RB is significantly dose dependent. Results further suggest that RB is selective for malignant cells since the concentrations tested on cancer lines did not induce death on non-malignant control cells. The mechanism involved in RB-induced cell death remained to be fully understood.

A 10% RB aqueous solution, named PV-10[®], is clinically tested as anticancer therapy following intralesional injection. PV-10[®] demonstrated to induce a first local effect of chemical ablation of the tumor and a second systemic effect in lesions not directly treated due to the onset of a tumor-specific immune response [6,42–44] (Figure 3). Eric Wachter et al [45] studied RB distribution in mice with an implanted liver tumor using the multiphoton microscopy technique. RB is demonstrated to localize in the extracellular environment in healthy tissue, while in tumor tissue, RB localizes intracellularly; specifically, it accumulates in lysosomes. Furthermore, short irradiation at 532 nm of RB already internalized in the diseased cells triggered rapid cell necrosis following lysosomal enzymes' release. The onset mechanism of the subsequent tumor immunity is not known in detail; however, the study published by Liu et al [44] showed that tumor necrosis induced by RB leads to the release of High Mobility Group Box 1 (HMGB1), which allows the activation and maturation of dendritic cells and the activation of tumor-specific T cells, so the activation of the immune response.

Provectus Biopharmaceuticals is a clinical-stage biotechnological company currently investing in RB and halogenated xanthenes to develop immunotherapy medicines that may be applied in different areas, including dermatology, hematology, virology, microbiology, and ophthalmology.

Nevertheless, to date results shared by Provectus mainly concern oncological research. The company has synthesized a version of RB free of typical commercial version impurities (US patent numbers are 8,530,675, 9,273,022, and 9,422,260), representing the active substance of PV-10®. The search on ClinicalTrial.gov including the terms "tumor" and "PV-10®" found 11 studies (updated to Jan 30, 2021), eight considered relevant for this topic: PV-10® is tested to treat neuroendocrine tumors metastatic to the liver (NCT02693067), breast cancer (NCT00237354), cancer of the liver (NCT00986661), and melanoma (NCT00521053; NCT00219843, NCT02288897); besides, PV-10® in combination with Pembrolizumab is tested to treat metastatic melanoma (NCT02557321). A study about the comparison of Rose Bengal and Toluidine Blue staining to early detect dysplasia in oral premalignant lesions is also reported (NCT03031899). Rose Bengal Disodium has been designated an Orphan Drug by FDA for the treatment of metastatic melanoma (12/21/2006), hepatocellular carcinoma (04/18/2011), neuroblastoma (11/02/2018), and ocular melanoma (02/11 / 2019).

3.3 Antimicrobial agent

RB application in the antimicrobial field, and the other hydroxyxanthenes, is currently limited even if their properties have been known since the second half of the nineteenth century (Figure 3). RB is a selective aid in microbiological media, i.e., Rose Bengal Chloramphenicol Agar is a selective medium for detection, isolation, and enumeration of yeasts and molds from food and for environmental monitoring of fungi [46,47]. RB has proved to be effective in the inhibition of bacterial growth. A recent study by Ferreira et al [12] evaluated *in vitro* the antibacterial effect of commercially available sterile ophthalmic strips containing three different dyes against selected Gram-positive and Gram-negative bacteria. Results provided that all the dyes (fluorescein, lissamine green, and rose bengal) showed antibacterial activity against Gram-positive bacteria and that preservative-free RB and lissamine green inhibited the growth of both Gram-negative and Gram-positive organisms. Nakonechny [48] and collaborators studied the antibacterial property of RB against *S. Aureus*, detecting that the minimum inhibitory concentration (MIC) of RB was 0.125 mg/mL. A combination of RB (0.008 mg/mL) and gentamicin was also tested, and it emerged a significant decrease in the gentamicin MIC from 0.005 mg/mL to 0.0016 mg/mL, suggesting a synergistic effect between the action of RB and the antibiotic. Considering that gentamicin acts by inhibiting protein synthesis and the process depends on the permeability of bacterial membranes, the authors suggested that RB facilitated the antibiotic's penetration into *S. Aureus* cells. Also, the negative charges of the dye stimulated gentamicin uptake by increasing the membrane potential as

the negative potential allows gentamicin to enter the cell. The mechanism by which hydroxyxanthenes act as antimicrobial is not clear; it can be hypothesized that halogens' presence contributes to their efficacy. Thanks to the lipophilic tendency, hydroxyxanthenes typically localize in the cellular membranes and can damage the microorganism at this level. Besides, they can directly bind and inactivate protein and enzymes: RB showed to bind and inhibited DNA polymerase, RNA polymerase, and NAD⁺ and NADP⁺ dehydrogenases [47]. RB has been demonstrated to reduce Herpes Simplex Virus-1 infectivity, probably inhibiting viral DNA polymerase [10,11].

The National Center for Advancing Translation Science (NCATS) has recently developed an open science data portal, named OpenData portal, to support the rapid development of therapeutics against SARS-CoV-2 [49]. Over 10.000 compounds are being tested, including RB. RB appeared to be active in disrupting the binding between SARS-CoV-2 Spike protein and host ACE2 receptor protein, which eventually leads to viral entry in host cells; furthermore, RB proved active in inhibiting the papain-like protease 3CL, a promising target to block viral replication (NCTAS ID: NCGC00159508).

3.4 Photodynamic and sonodynamic therapy.

Other characteristics of RB are its sono-photodynamic activity (Figure 4). RB as a PS drug first appeared in the research article published by Schmidt and Norman in 1920 [50], in which the object of the investigation was to determine the cause of the inability of gelatin to protect red cells against the photodynamic action of eosin, a xanthene dye as RB. PDT is a medical treatment that involves three essential components: tissue oxygen, a light source, and the PS; PDT has been successfully used to treat skin and ocular disorders, as well as antimicrobial therapy and cancer; in particular, it is approved as first-line treatment for keratinocytes skin cancer. PDT procedure consists of three phases: first, PS is administered locally or systemically and it accumulates selectively in abnormal cells; second, a light source of an appropriate wavelength irradiates the target tissue and photochemically activates PS, which, in the last phase, damages the target cells leading to cell death [14,15,24–26]. To cause damage, PS can act through two mechanisms, type 1 or type 2, and consequently, this allows to identify them as type 1 or type 2 PS; RB acts preferentially via type 2 mechanism: once activated, it transfers its energy directly to oxygen in the ground state (3O_2) leading to the ROS singlet oxygen (1O_2), which in turns reacts with molecules in its radius of action to lead to cell damage, in particular membrane destruction and enzymatic inactivation [16,17]. Based on PDT, since 1989, a parallel and complementary therapy with the same outcomes and

procedure phases has been investigated, the sonodynamic therapy (SDT): the difference with PDT is that the damage to the target tissue is given by irradiation by an ultrasonic wave instead of a light source, and the molecule mediating the therapeutic effect is called sonosensitizer drug (SS) [51,52]. The mechanism of action of SDT is not clear, but the cavitation effect seems to be the main responsible for cell destruction. Through the cavitation effect, ultrasounds generate pressure variations in the interstitial fluid leading to a rapid formation of bubbles which increase and decrease their volume until a collapse, this implosion is directly able to destroy the cell membrane, and if the formation of bubbles occurs inside the cell, also the cytoskeleton is destroyed. The cavitation effect can also trigger the emission of sonoluminescence and the temporary heat generation (pyrolysis) phenomena, responsible for ROS production [17,51,53]. RB can induce ultrasonic cavitation, and it has been tested both as an enhancement for RB-PDT and alone [15]. To date, there are two significant limitations in the PDT/SDT context, the photosensitivity phenomena and the tissue penetration depth of light. Photo-sonosensitivity phenomena are due to the lack of selectivity of the sensitizer, which can be activated by external stimuli even if it has not reached the target tissue causing undesirable effects. The tissue penetration depth depends on the wavelength at which PS is excited: UV-visible light penetrates the tissue up to a maximum of 3 mm and may be insufficient to treat deeper tumor lesions. On the other side, NIR light is demonstrated to penetrate tissues deeper, but many more endogenous chromophores can absorb it, causing severe biological damages; thus, light "therapeutic window" results in between 650 – 900 nm [54–56].

RB mediated PDT can eradicate bacteria strains [14,57,58], the photodynamic antimicrobial chemotherapy (PACT) represents a promising solution to antibiotic resistance. RB-PACT was demonstrated to inhibit antibiotic-sensitive and resistant strains of Gram-positive *S. Aureus* and Gram-negative *P. Aeruginosa*, and the combination of RB-PACT and methicillin significantly decreased the MIC of methicillin for *S. Aureus* strains [59]. RB-PDT has eradicated infections secondary to fungal species: RB successfully inhibited *Fusarium keratoplasticum* species *in vitro* and in a patient suffering from fungal keratitis following two sessions of treatment [60]. The efficiency of RB-PDT on candidiasis and onychomycosis has also been reported [61,62]. The sonodynamic antimicrobial chemotherapy (SACT) can also be considered a potential antimicrobial strategy. Nakonechny et al [52] first evaluated RB-SACT on the Gram-positive *S. Aureus* and Gram-negative *E. Coli* in a closed dark chamber of an ultrasound bath (ultrasound frequency 28 kHz, intensity 0.84 W cm⁻²). RB-SACT inhibited both bacteria, the maximum inhibition was registered at 5 µM and 15 µM for *S. Aureus* and *E. Coli*, respectively. The higher sensitivity of Gram-positive has also been

observed for RB-PACT [14], and it can be related to the structural differences of the cell membrane of the two bacteria group. The more permeable outer cell wall of Gram-positive bacteria allows PS to translocate to the plasma membrane efficiently. Increasing the permeability of the Gram-negative membrane would allow PS to penetrate to sensitive cellular locations, and it appeared that pronounced cationic charges could represent a solution [63].

RB mediated PDT has been studied on several cancer cell lines, including oral cancer cells, HeLa cells, gastric adenocarcinoma cell line, and breast cancer cells [17,21,64–66] with promising outcomes. RB-PDT has also been investigated on melanoma treatment, demonstrating its efficacy in decreasing cell proliferation even under sunlight exposure [67]. McEwan et al [68] demonstrated that RB-SDT is significantly more effective than RB-PDT in B16 melanoma: after irradiation of 60 seconds, RB-SDT reduced cell viability by 40% while RB-PDT decreased only by 18%. This difference is due to the dark pigmentation of the melanotic lesion, which acts as a filter for the penetration of light and to the natural antioxidant action of melanin which lowers the efficacy of PDT; moreover, ultrasounds can penetrate deeply in the tissue compared to light [53,68].

RB, along with riboflavin, has been identified as the most promising photoactive dye for photochemical tissue bonding (PTB), a sutureless tissue repair technique for wound healing and scar formation. In this case, RB represents a photosuturing agent applied via aqueous solution (typically 1 mM) on the tissue's surfaces. The tissue exposure to green wavelengths triggers ROS production, promoting the formation of covalent crosslinking between the structural proteins on the two tissue edges. PTB can minimize scar formation and fibrosis typical of standard suture techniques, and, contrary to laser tissue welding, thermal damages are avoided since no heat application is needed [17,69,70]. RB-PTB benefits have been observed in different areas to repair tissues (i.e., ophthalmology, neurology, gastroenterology, vascular surgery) as detailed reviewed by Vanerio et al [17] and Redmond et al [70].

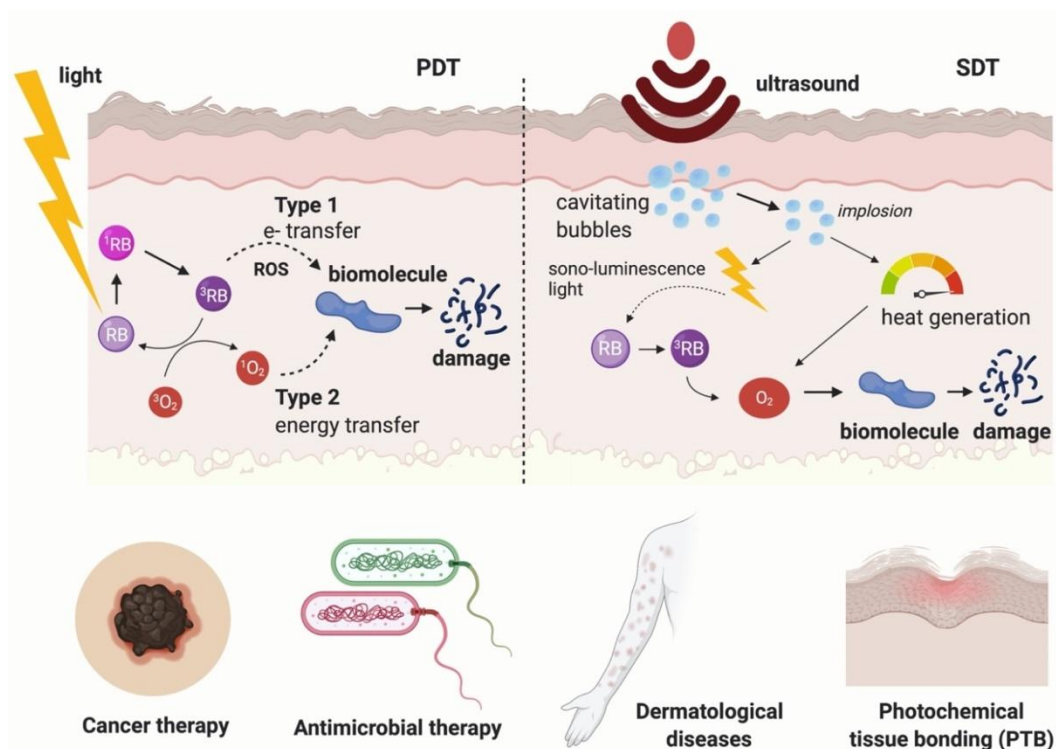


Fig. 4. Rose Bengal application by PDT and SDT. PDT: upon green light irradiation, RB at the ground state is excited to ^3RB which can react via type 1 or type 2 process; Type 1: ^3RB directly reacts with biomolecules in its radius of action causing cell damage, Type 2: ^3RB reacts with tissue oxygen which in turns reacts with biomolecules causing cell damage. SDT: exposure to ultrasounds generates the cavitating bubbles that implode generating heat and triggering the emission of photoluminescence light which can excite RB; in both cases, a further reaction with tissue oxygen provokes biological damage. Application: PTD and SDT can be applied, alone or in synergism, in cancer therapy, antimicrobial therapy, dermatology/aesthetic field; Light irradiation is exploited also in the photochemical tissue-bonding (PTB). Created with BioRender.com

4. Rose Bengal Delivery and RB nano-based delivery systems.

4.1 Rose Bengal derivatives.

RB structural changes have been proposed both for the benzoic substituent and for the xanthene ring. As previously mentioned, the xanthene ring plays the leading role for RB photosensitive properties, and permanent changes could negatively affect them. In contrast, the benzoic ring's chemical modification probably would not influence nor the absorption/emission spectra neither ROS production. However, the insertion of hydrophobic acetate groups on the xanthene ring has also been proposed because, once inside the cell, cytoplasmatic carboxylesterase removed the acetate groups restoring the original structure of RB without affecting its properties [16]. Pereira et al [23] synthesized three hydrophobic RB ester derivatives adding hydrocarbon chains of different lengths to the benzoic ring (Figure 5). The improvement of RB hydrophobicity via the pharmaceutical

chemical approach has the advantage of directly solving poor cell crossing, leading to increased RB intracellular uptake. On the other side, the increased intracellular uptake in this way would not be cell-selective, causing different RB biodistribution and increasing drug accumulation in non-target cells, site-specificity would be lost, and the possibility of side effects would be higher [22]; in conclusion, therapeutic effect and safety would be compromised.

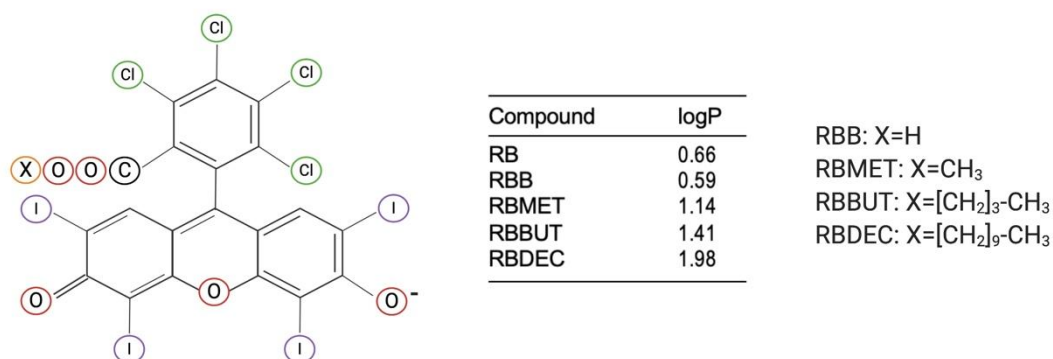


Fig. 5. RBB derivatives synthesized by Pereira et al [23] and partition coefficients. Created with BioRender.com.1

4.2 Rose Bengal Delivery Systems.

A technological investigation for designing suitable RB delivery systems is essential to accomplish a safe and effective therapeutic impact based on previous considerations. Table 2 resumes nano-based RB delivery systems already studied.

<i>Type of nanocarrier</i>		<i>System</i>	<i>Size</i>	<i>Target</i>	<i>Findings</i>	<i>Ref.</i>
Inorganic	Gold	electrostatic and covalent AuNPs-RB nanocomplex	53 nm; 49 nm	PDT, Vero, and HeLa cell lines	↑ cytotoxicity in HeLa cells vs. Vero cells ↑ phototoxicity	[71]
		Gold nanorods conjugated with RB	13 nm	PDT and PTT, Cal-27 cell line	↑ intracellular uptake ↑ PDT-PTT efficiency vs. free RB	[21]
	Silica	Amino mesoporous silica-RB nanoconjugate	160-180 nm	PDT, SK-MEL-28	Protective effect of NPs towards RB photodegradation	[72]
		Amino mesoporous silica-RB nanoconjugate	500 nm	aPDT, C. Albicans	↑ intracellular uptake ↑ aPDT efficiency vs. free RB	[73]
		Electrostatic and covalent SiNPs-RB nanocomplex	50 nm; 35 nm	PDT, MCF-7 and 4451 cell lines	↑ intracellular uptake, highest for covalent complex ↑ phototoxicity, highest for covalent complex	[64]

	Gadolinium Oxide	Dextran coated Gd ₂ O ₃ NPs carrying RB	17 nm	Magnetic resonance and optical imaging	Slow releasing system ↑ intracellular uptake <i>in vitro</i> optical tracking	[74]
Polymeric	Chitosan	RB functionalized chitosan NPs	-	aPDT, E. faecalis, stabilization of dentin-collagen matrix	↑ uptake ability ↑ death of bacterial cells ↓ dentin-collagen degradation	[75]
		Chitosan/RBD self-assembled NPs	150-200 nm	Hep42, HT29, CT-26; SDT	↑ uptake ability ↑ selective tumor accumulation	[22]
	PLGA	PLGA/RB colloidal particles	246-248 nm	HCC70	↑ cytotoxicity	[65]
	PGA	PGA-RB derivative nanoparticles	160 nm	PDT, xenografted Bcap37 breast tumor	↑ phototoxicity ↑ tumor accumulation ↑ blood circulation time	[76]

					↑ antitumor efficacy	
<i>Lipid-based</i>	Hydrogel of RB loaded PC/Chol liposomes	-	PDT, IPL; light hair removal	RB migration in dermal layer ↑ decrease in hair density	[77]	
	Hydrogel of RB loaded PC/DPPC/Chol/TP MLV	2.1-4.2 μm	topical RB delivery	↑ encapsulation efficiency decreasing particle size ↑ RB delivery into deeper layers	[78]	
<i>Dendrimers</i>	Complex of cationic phosphorus dendrimers and RB	-	PDT, BCC cell line	↑ singlet oxygen production ↑ cell uptake and cell uptake over time ↑ phototoxicity ↑ apoptotic and necrotic cell death ↑ ROS production	[79,80]	
	PAMAM dendrimers encapsulating RB	20 nm	PDT, Dalton Lymphoma Ascites (DLA) cell line	↑ phototoxicity	[31]	

<i>Upconversion NPs</i>	PAAm modified upconversion NPs/ hyaluronate- RB	459.9 nm	PTB	↑ biocompatibility and penetration due to HA ↑ penetration due to NIR light fastest and stronger tissue bonding	[81]
	oleogel incorporating white light-emitting NaYF ₄ UCNPs conjugated with RB	-	RB transdermal delivery, PDT	↑ penetration of oleogel vs. free UCNPs ↑ ROS production of oleogel vs. free UCNPs	[82]
<i>Nanocapsules</i>	self-assembled polypeptide nanocapsules loading RB	17.28 nm	PDT and SDT, superficial malignant tumor, B16, and HeLa cell lines	↑ cell uptake ability ↑ ROS generation tumor cell elimination tumor lesion necrosis	[83]
<i>Exopolysaccharide NPs</i>	self-assembled NPs of EPS-RB	35.2 nm	PDT, Gram+, and Gram-	↑ singlet oxygen generation ↑ cell uptake ability ↑ photodynamic inactivation of bacteria	[84]

Microcarriers	RB α -cyclodextrin microparticles	1-8 μm	PDT, streptococcus mutans	$\uparrow$ photodynamic inactivation of bacteria	[85]
	RB Microbubbles converted in situ into RB NPs	100 nm	SDT, theranostic, HT-29	$\uparrow$ singlet oxygen generation $\uparrow$ cell uptake. $\uparrow$ efficacy in killing tumor cells	[86]

Table 2. RB nano and micro-based delivery system.

4.1.1 Inorganic nanoparticles.

Inorganic NPs are particles made of metal oxides (i.e., titanium, gadolinium, iron) or metallic composition (gold, silver, silica) that are developing both as intelligent drug delivery systems and as imaging contrast agents [87,88].

4.1.1.1 Gold NPs.

Gold NPs are very low cytotoxic carriers characterized by high stability and the possibility of accurately controlling shape and size; the covalent association and non-covalent conjugation between drugs and ligands with the NPs surface provide efficient drug loading and site-specificity [71,87].

Prasanna et al [71] synthesized gold NPs capped with glutathione (GSH) electrostatically and covalently bound to RB to obtain AuNPs-RB nano complexes to improve RB PDT efficacy. AuNPs-RB nano complexes phototoxicity and dark toxicity were tested versus free RB on Vero and HeLa cell lines, respectively, kidney epithelial cells from African green monkey and cervical cancer cells. The electrostatic complex was not stable due to the fragile binding nature harming intracellular uptake and, consequently, its cytotoxicity. On the other side, the covalent complex showed higher cytotoxicity resulting in being twice as efficient as free RB. According to the literature, the authors suggested that the strong covalent binding improves the intracellular uptake.

Wang et al [21] synthesized gold nanorods (GNRs) conjugated with RB. Gold nanorods are rod-shaped NPs easy to synthesize with properties in the near-infrared (NIR) region that demonstrated prolonged blood circulation. The authors applied RB-GNRs to PDT and photothermal therapy (PTT) as oral cancer treatment, testing them on Cal-27, human oral squamous cell carcinoma cell line, and *in vivo* in hamster cheek pouch grew tumors. Results revealed that the uptake of RB-GNRs by cells is improved compared to free RB uptake with accumulation in oral cancer cells due to the specificity of RB for oral cancer cells, explaining the increased efficacy of the combined PDT-PTT treatment, which has demonstrated to be minimally invasive and non-destructive.

4.1.1.2 Silica NPs.

Silica NPs, have gained scientific interest as drug carriers because of the facile synthesis and the versatility of drug incorporation and surface functionalization, indeed they can incorporate inorganic material within or on the surface of the structure and a wide range of molecules can be linked to silica surface thanks to flexible silane chemistry. Besides, SiNPs are biocompatible and stable carriers with prolonged blood circulation due to their hydrophilic surface [30,64].

Uppal et al [64] conjugated RB to organically modified silica NPs by electrostatic or covalent interaction studying their phototoxicity vs. free RB against 4451 and MCF-7, respectively, oral and breast cancer cell lines. Under light irradiation, RB-SiNPs covalent complex resulted in being the most cytotoxic against both cell lines. The enhanced phototoxicity is due to the higher cell uptake; moreover, the covalent interaction between RB and SiNPs increases the complex's photostability and the singlet oxygen generation.

Another emerging nanocarrier is mesoporous silica nanoparticles (MSNs), which exhibit a higher surface area thanks to many empty porous able to entrap active biomolecules [88]. Gianotti et al [72] and Paramanantham et al [73] conjugated RB to amine-functionalized MSNs to assess its phototoxic properties against SK-MEL-28 cell line (melanoma cellular model) and in the antimicrobial PDT (aPDT) framework to eradicate *C. Albicans*, respectively. Gianotti et al [72] covalently attached RB to amine-functionalized MSNs and tested them vs. unloaded MSNs on a melanoma model. Tests demonstrated that RB confined inside the particles is successfully excited under green light with an efficient singlet-oxygen production representing a promising tool for melanoma eradication. Moreover, the authors demonstrated that nanocarriers could protect RB from premature photodegradation. In the study of Paramanantham et al [73], RB was bound to MSNs through the electrostatic interaction between the positive charge of amine and the negative charge of the drug; moreover, the amine groups in the particle pores allow the slow and controlled release of RB, fundamental in aPDT context to decrease the incubation time. The cellular uptake was maximum for MSN-RB compared to free RB and the antimicrobial activity, loading capacity, and RB drug release efficiency.

4.1.1.3 Metal-oxide NPs.

Metal-oxide NPs are multifunctional particles mainly employed in the theranostic field, the contemporary diagnosis, and therapy of a pathological condition. To maximize diagnostic application, metal-oxide NPs were conjugated with fluorophores to combine magnetic resonance imaging (MRI) with fluorescence imaging (FI), raising the possibility to get highly resolved three-dimensional images of the body with tracking through fluorescence microscopy [74,89]. Kumar et al [74] synthesized dextran-coated Gd₂O₃ NPs carrying RB for magnetic resonance and optical imaging; moreover, they performed *in vitro* studies on cancerous and healthy cell lines to assess cell uptake ability and cytotoxicity of nanocomplexes. As gadolinium-based contrast agents report mild side-effects and gadolinium can accumulate in the body, NPs were coated with dextran to assure biocompatibility and no-toxicity. The ¹H NMR line broadening effect and the fluorescence

microscopy images of NPs revealed that they could be efficiently used as contrast agents; the intracellular uptake and the optical activity demonstrated an efficient *in vitro* optical tracking with a lack of cytotoxicity.

4.1.2 Organic nanoparticles

4.1.2.1 Polymeric NPs.

Polymers are widely used in nanosized drug delivery thanks to their advantages of high biocompatibility and biomimetic characteristics; most of them are also biodegradable resulting in safe metabolites. A characteristic feature of polymeric NPs is the possibility of slow and controlled drug release as the result of their erosion and degradation, in this way, drug half-life and *in vivo* particle circulation are improved. Moreover, polymeric NPs are easy to design and not expensive to synthesize with a facile control over shape and size; polymers commonly used include chitosan, albumin, dextran, hyaluronic acid (HA), poly-lactic-co-glycolic acid (PLGA) [87,90–92].

4.1.2.2 Chitosan NPs.

Chitosan (CS) is a cationic polyamine constituted by copolymers of glucosamine and N-acetylglucosamine due to partial deacetylation of chitin, the most abundant biopolymer in nature after cellulose. Thanks to excellent biodegradability and biocompatibility, CS has been investigated in drug delivery as the main constituent of a nanocarrier, coating agent, mucoadhesive compound, and a disgregant and binder [75,93].

Shrestha et al [75] have synthesized RB functionalized CS NPs (CSRBnp) employing the interaction between cationic charges of CS and anionic charges of RB; the study aimed to assess the antibacterial/antibiofilm efficacy of CSRBnp PDT against *E. faecalis* and the dentin-collagen stabilization ability. Results revealed a higher uptake of CSRBnp by bacterial cells and biofilm and a slower release of singlet oxygen vs. free RB due to the conjugation of RB to CS and nanosized complexes. As a consequence of higher uptake, both in the dark and under green light irradiation, bacterial membrane damage and leakage of cellular constituents have been observed, and the exposure to PDT killed most of the bacterial cells. Moreover, exposure to PDT reinforced the collagen matrix structure by increasing the number of anionic reaction sites, hence the number of ionic complexes between CS and collagen.

Gao et al [22] loaded CS NPs with RB ester derivative RBD (ω -carboxyheptil ester) to passive target tumor tissue and to efficiently transport the nanocomplex into tumor cells. CSRB NPs and CSRBD NPs have been tested on human liver hepatocellular carcinoma cell line (Hep42), human

colon carcinoma cell line. Investigation through fluorescence microscopy revealed that cell uptake of free RBD was higher than free RB thanks to the alkyl chain; on the other side, the uptake of both CSRBDs and CSRBs was higher than the free forms with the maximum uptake for CSRBDs. Tumor accumulation was studied in colon cancer transplanted mouse model after intravenous administration. Results revealed that RBD biodistribution was not tissue-specific, contrary to RBDCNs which accumulated more in lung-specific colon cancer metastases than other organs. The authors concluded that selective accumulation and improved uptake make this nanocomplex suitable for targeted sonodynamic therapy.

4.1.2.3 PLGA NPs.

PLGA is a family of biodegradable and biocompatible polymers constituted by a copolymer of poly-lactic acid (PLA) and poly-glycolic acid (PGA). PLGA has been widely investigated as carriers in drug delivery systems, and the FDA has also approved it for this purpose. PLGA provides slow and controlled release of drug thanks to its erosion time; moreover, it is hydrolyzed in the body in the original monomers which are endogenous, therefore, it presents a complete lack of toxicity [87,94].

Loya-Castro et al [65] incorporated RB into PLGA colloidal particles (CPs) and tested them on HCC70 human triple-negative breast cancer cell line, carcinoma grade 3. CPs have been synthesized by two methods: encapsulating RB in the PLGA CPs (PLGA/RB CPs) and by electrostatic adsorption of RB on chitosan-coated PLGA CPs (PLGA/Ch/RB CPs). As PLGA/RB CPs showed slow drug release kinetic due to the low diffusivity of RB within the polymeric structure, PLGA CPs particles have been coated with chitosan to let its positive charges interact with negative charges of RB, and a faster release kinetic has been observed. Cytotoxicity tests revealed that RB causes cell death by necrosis, probably due to lysosomal damage, and inhibition of cell viability about 50%. The same authors have planned further studies to assess the efficacy of the nanosystem in cancer therapy.

Zhang et al [76] conjugated a derivative of RB onto biocompatible PGA to obtain self-assembled PGA-RB nanoparticles to assess the antitumor efficacy on xenografted Bcap37 breast tumor. Results demonstrated that PGA-RB NPs were 40 times more toxic than free RB under green light irradiation, besides blood time circulation and tumor cell accumulation were improved with the consequence of an increased efficacy as an anticancer therapy.

4.1.2.4 Liposomal carriers.

Liposomes are spherical artificial vesicles structured by an internal aqueous core and an external hydrophobic bilayer made of phospholipids or cholesterol. To improve stability or pharmacokinetic characteristics, like blood circulation time or selective cell uptake, surfactants can be added and

functionalized or coated with other molecules. The main advantage of liposomal carriers is the dual nature of hydrophilic/hydrophobic carriers since both water-soluble and no water-soluble drugs can be loaded, the first one into the core and the second one in the external bilayer [87,91]. As RB is an amphiphilic photosensitizer (PS), it may be assumed that it would distribute in both compartments if loaded into a liposome.

Samy et al [77] evaluated the efficacy of PDT using the topical gel of liposomal RB followed by intense pulse light (IPL) to remove grey and white hair. Laser and IPL epilation require a chromophore absorbing its energy to be effective; in dark hair, melanin is exploited as an endogenous chromophore, but it is absent in light hairs: in this study, RB has been used as exogenous chromophore instead of melanin. RB liposomes (RBL) have been developed using phosphatidylcholine and cholesterol as lipids, and successively RBL suspension has been gelled to obtain the final topical hydrogel. This RB delivery system has been evaluated *in vivo* on healthy albino mice and in a clinical study. Skin permeation study on albino mice revealed the penetration of RBL in all epidermal layers through fluorescence imaging; after IPL exposure, damaged and necrotic hair follicles were observed in the dermal layer while the stratum corneum remained intact as RB migrated into deeper layers. The clinical study showed that the decrease of hair density in 12 weeks after 3 treatments sessions was 44% in the treatment group and 16% in the placebo control group; the reduction 6 months after the last treatment was 40% in the treatment group and 11.6% in the control group. The study demonstrated that RBL selectively delivers RB in the deeper dermal layer and RB PDT-IPL treatment could be useful for light hair removal, but further studies are required.

Maha Fadel M. Ali [78] investigated RB delivery into skin layers for topical PDT loaded into different multivesicular liposomes (MVL) in hydrogel formulation. MLV were prepared using phosphatidylcholine (PC), dipalmitoylphosphatidylcholine (DPCC), cholesterol (CHOL), and tripalmitin (TP), successively two carboxy-methylcellulose (CMC) hydrogel have been prepared, one containing free RB (F-RB) and one containing loaded MLV. A released study has been performed; results showed a slower RB release from MLV-RB hydrogel than F-RB due to the number of barriers RB meets, such as liposome layers and then the hydrogel matrix. On the other side, following hydrogel's application, free RB did not penetrate beyond the epidermis due to its primary water-soluble nature, while hydrophobic RB-MLV allowed RB migration into deeper skin layers.

4.1.2.5 Dendrimers.

The term dendrimer has its roots in the Greek word "dendron" meaning tree, which entirely gives the idea of the defined architectural motif of these nanosized range particles. Dendrimers are globular highly branched macromolecules consisting of layers of branching units, called dendrons, growing from a central core; each layer forms a generation, and the molecule becomes more branched as the generations grow. The most external layer is usually functionalized with peripheral chemical groups which determine the dendrimer chemical behavior, as a consequence, they determine the nature of carrying drugs and the drug targeting ability. Drugs can be attached to the peripheral groups, covalently bound to the core or the dendrons, and encapsulated in the internal cavities. The advantage of using dendrimers as DDS relies on the possibility of controlled delivery of drugs and it has been emerging as a personalized medicine tool [79,95,96].

Dabrzalska et al [79,80] first investigated the ability of positively charged phosphorus dendrimers to form complexes with RB, oppositely charged, via spectroscopic analysis. Subsequently, they investigated the cationic dendrimer-RB complex RB-1cat properties to assess its potential utility in basal cell carcinoma (BCC) photodynamic therapy. Fluorescence studies revealed an increased singlet oxygen production of RB-1cat vs. free RB or 1cat, detected as an increase of RB-1cat fluorescence over the time of irradiation reaching a plateau after 20 minutes. Further studies were conducted in-vitro using three BCC murine cell lines. The cellular uptake ability of free RB and RB-1cat was tested exploiting RB fluorescence monitored by flow cytometry. Higher uptake of RB-1cat has been observed; RB-1cat uptake increased over time while free RB uptake was constant. To determine RB-1cat phototoxic ability, the absence of dark toxicity was firstly demonstrated, second the highest phototoxicity, ranging from 85% to 95%, of RB-1cat was observed against all three cell lines. The phototoxicity was due mainly to the apoptotic cell death mechanism, which was more relevant than free RB and the ROS production. Karthikeyan et al [31] synthesized polyamidoamine (PAMAM) dendrimers encapsulating RB (G2.5 PAMAM+RB) to test them on Dalton Lymphoma Ascitis (DLA) cancer cell lines in the PDT framework. The spectroscopic and fluorescence analysis conducted on G2.5 PAMAM+RB revealed that RB is both encapsulated into dendrimers cavities via electrostatic interaction and also absorbed at the terminal surface; drug loading was also measured spectrophotometrically demonstrating that RB was efficiently loaded into dendrimers and the release kinetic, measured over 72 hours, resulted in being faster in the initial hours. Following the demonstration of the absence of the dark toxicity of G2.5 PAMAM+RB and the demonstration of no modification in ROS production, phototoxicity has been tested, resulting in higher toxicity of G2.5 PAMAM+RB compared to free RB.

4.1.2.6 Upconversion nanoparticles.

Upconversion NPs (UCNPs) are a class of nanomaterials able of the photon upconversion process, which is the absorption of two or lower energy photons that are up-converted into one emitted high energy photon. The upconversion process relies on the fact that multiple intermediate states exist between the ground state and excited state, which can accumulate low-energy excitation photons in the near-infrared (NIR) light region subsequently emitted as one photon in the visible/UV region. Dopant ions in NPs are responsible for the upconversion efficiency, usually lanthanide or actinide [97,98]. The upconversion technology is mainly used in PDT to overcome UV light limits *in vivo*, such as the low tissue penetration that prevents PDT from treating deeper lesions and the possibility that UV-light can degrade many endogenous chromophores. On the contrary, NIR light has the best effectiveness of penetration tissue because it is not absorbed by endogenous chromophores that absorb UV light [54–56]

Han et al [81] synthesized polyallylamine modified UCNP/ hyaluronate-RB (UCNP/PAAm/HA-RB) for noninvasive photochemical tissue bonding on porcine tissue. Dark cytotoxicity of the conjugate has been investigated, and results revealed that conjugates without HA were more toxic than the others with HA, which maintained cell viability over 90%. Similar results were obtained testing dark cytotoxicity of RB alone vs. HA-RB conjugate, concluding that HA improves biocompatibility. Light delivery efficiency and UCNP/PAAm/HA-RB penetration were tested *ex vivo* into the closed incision of porcine skin, finding that green light was scattered in the stratum corneum while NIR light reached the dermal layer; moreover, UCNP/PAAm/HA-RB was much more detected in the tissue compared to UCNP/PAAm. The transdermal delivery was tested *in vivo* comparing both RB alone vs. RB-HA and the complexes UCNP/PAAm vs. UCNP/PAAm/HA-RB: similar results were obtained because in both cases the deepest penetration has been observed in the presence of HA, which behaves as a penetration enhancer. The photochemical tissue bonding efficiency has been tested *in vivo* in the dorsal skin of mice, showing that UCNP/PAAm/HA-RB under NIR light irradiation caused the fastest tissue bonding that was later assessed by tensile strength test demonstrating a higher tensile compared both to UCNP/PAAm/HA-RB without NIR and to conventional suturing.

Dhal et al [82] formulated an oleogel incorporating white light-emitting NaYF₄ (WEN) UCNPs conjugated with RB to deliver RB deeper in the dermis and efficiently generate ROS under NIR light irradiation on porcine skin. WEN UCNPs synthesized were hydrophobic and doped with Li⁺ ion obtaining LiWEN-RB UCNPs, subsequently formulated in oleogel of soybean oil using stearic acid as gelator (LiWEN-RB-G). The *in vitro* skin permeation experiments were conducted to compare LiWEN-RB vs. LiWEN-RB-G; results revealed that penetration was more efficient if NPs are incorporated in

the oleogel, probably because oleogel components create a transient pore in the stratum corneum acting as penetration enhancers. The same samples were subsequently subjected to ROS production, finding a gradual increase if UCNPs incorporated into gels. Further studies on skin segments under NIR irradiation revealed a significant ROS production underneath the skin, representing a potential application for more in-depth PDT cancer therapy.

4.1.3 Other carriers.

In addition to previously mentioned RB delivery systems, other types of carriers have been proposed.

Liu et al [83] developed a self-assembly nanocapsule loading RB, called PARN, by mixing an amphiphilic polypeptide PA (C18GR7RGDS) and RB to treat superficial malignant tumor by PDT and SDT. In this study, PA has the trifunctional role of tumor targeting, cell penetration, and amphiphilicity. PARN was tested in cytotoxicity, ROS generation, SDT, and PDT on tumor-xenografted nude mice. Tests performed on B16 and HeLa cell lines showed that cells efficiently internalized PARN and, consequently, cell viability decreased much more strongly compared to free RB, and the ROS generation inside the cells was higher. *In vivo* studies were performed exhibiting nude mice both to PARN + ultrasound and to PARN + ultrasound + laser after PARN intravenous administration; both appeared efficacious to harness tumor growth, and the analysis of organs after mice sacrifice confirm the efficacy in tumor cell elimination and tumor necrosis due to tumor vasculature damage without relevant side-effects.

Li et al [84] conjugated RB with bacterial exopolysaccharide (EPS) to generate the amphiphilic complex EPS-RB, which self-assembled into NPs in solution, to eradicate Gram-positive and Gram-negative bacteria photodynamically. *E. Coli* and *S. Aureus* were used as Gram-positive and Gram-negative bacteria models. It was observed a higher singlet oxygen generation for EPS-RB than free RB and a higher photoinactivation of bacteria after irradiation, meaning that less amount of EPS-RB is needed to eradicate bacteria vs. the amount of RB. Moreover, RB fluorescent tracking revealed that more EPS-RB accumulates into the cell than free RB with no toxicity toward healthy cells.

Alexandrino et al [85] developed and described RB α -cyclodextrin microparticles and tested them to eradicate *Streptococcus Mutans* by PDT for preventing dental caries. RB encapsulated into microparticles allowed to eradicate bacteria with a concentration 10 times smaller than free RB, probably due to a higher cell intake through bacterial membranes.

Microbubbles (MBs) are core-shell gas-filled microspheres with a maximum diameter of about 10 μm particularly useful in diagnostic medical sonography because of their ability to differentiate

between tissue and blood vessels once injected intravenously and submitted to ultrasounds. In the last decades, MBs are being investigated as drug delivery carriers due to the advantage that, when irradiated by ultrasounds, they can disrupt and release the drug. The principal limit of MBs is its diameter since a smaller size (1-5 μm) is required to leave the blood vessels and distribute in tissues [99]. Based on this, Hou et al [86] designed RB microbubbles (MBs) that can be converted into RB NPs after exposing them to local ultrasound to improve SDT efficacy in treating cancer; human colorectal adenocarcinoma cell line (HT-29 cells) has been employed for *in vitro* tests. First, RB amphiphilic derivative (ARB) was synthesized and used to prepare the shell of MBs encapsulating a core of C3F8 gas (PFC). Subsequently, MBs were exposed to 1minute ultrasound to in situ convert them in NPs. The evaluation of singlet oxygen generation showed that sonicated MBs increased the production of singlet oxygen probably due to MBs collapse; the cellular uptake test evaluated by fluorescence techniques revealed a higher uptake if MBs are subjected to the US because the US induces enhancement of cell membrane permeability and because of the conversion to NPs. *In vitro* cytotoxicity was evaluated both in healthy human cells and in HT-29 cells; no cytotoxicity has been shown towards healthy cells, demonstrating proper biocompatibility. Regarding HT-29 cells, it was observed that only RB-MB and RB-NP exposed to the US could efficiently kill tumor cells with a better SDT therapeutic efficiency. The unique advantage of RB-MBs compared to other systems previously described is that they ultrasound contrast agents, so they can be used in diagnostic imaging. *In vivo* ultrasound imaging was examined in a white rabbit: a clear echo signal was detected in the renal cortex, and RB-MBs were also seen to cross pulmonary capillary to reach the systemic circulation. Same tests on mice bearing HT-29 tumors demonstrated that the tumor site was rapidly detected. All these data suggest a potential and effective use in theranostic.

5. Discussion and perspective

The focus of the current review is to determine if nanosized delivery systems can represent a tool to improve RB-based therapy. Drug delivery science aims to deliver a drug in a targeted manner sparing healthy tissues and controlling both release rate and release location. In the absence of a proper delivery system, a drug can suffer from low bioavailability, instability, and plasma level variation, making repeated administration necessary to reach the therapeutic effect. Side effects can be more relevant due to the impossibility of controlled release location and the hepatic first-pass effect. For this purpose, nanoparticles have shown to be promising carriers due to unique physical-chemical and biological properties.

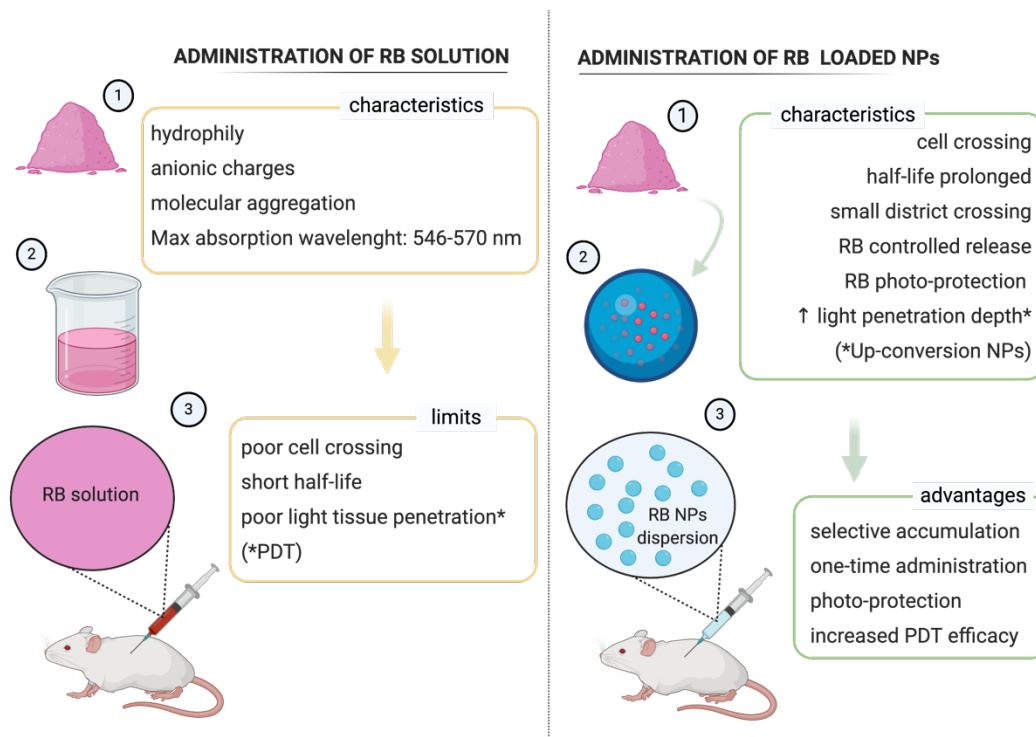


Fig. 6. Administration of free RB versus administration of RB loaded NPs. RB is a water-soluble drug with a short half-life; when administered without a proper delivery system it suffers from short circulation time, poor biodistribution and cell crossing needing a multiple and intralesional administration to reach a therapeutic effect; moreover, RB is activated by green light with a limited tissue penetration depth (PDT). RB loading into NPs increases cell crossing and half-life, protects RB from undesired light activation and degradation, controls RB release in time and body districts with the advantages of one-time administration, other administration routes are considered. Upconversion NPs can improve the light penetration depth. Created with BioRender.com.

The main advantage of nano-based delivery systems is their nanosized range (around 100 nm), which gives them the ability of precise drug delivery to their intended target, avoiding no specific systemic release. Properly designed NPs can also solve solubilities and hydrophilicity problems based on material choice and NPs surface functionalization. Previously, limitations of free RB such as poor cell uptake, short half-life and scarce tissue penetration in PDT/SDT context have been discussed; however, literature addresses the ability or potential ability, of the various NP approaches to overcome these limitations (Figure 6).

RB liposomal carriers proved to overcome issues related to RB water-solubility and poor lipophilicity, crucial features to assure dermal delivery of drugs; indeed, RB liposomes enhanced RB delivery up to dermal layers whereas free RB did not penetrate beyond the epidermis [77,78]. Similarly, the formation of NPs conjugate with the drug may to enhance the drug uptake even for hydrophilic substances like RB, [87,90–92,100]. Herein, examples of RB-NPs conjugates have been reported demonstrating to increase RB cell uptake ability. Covalent complexes are the most suitable to improve RB intracellular uptake than electrostatic ones as described for silica and gold NPs); indeed, the non-covalent interactions between RB and the NPs appeared to be momentary and

unstable, resulting in a poor uptake [64,71]. RB has two negative charges at physiological pH hampering the interaction with negatively charged cellular structures or Gram-negative bacteria, a phenomenon supposed to further limit RB uptake by cells; encapsulating or conjugating RB to positively charged nanocarriers proved to be a promising approach in this sense. RB-chitosan NPs showed an increased cell uptake into bacteria cells, and the authors mainly attributed this finding to positively charged carrier and nano dimension since they increase the reactivity with other charged structures facilitating the dye penetration [75]. Similar outcomes on cancer cells resulted in synthesizing RB-cationic phosphorus dendrimers [80]. Nanotechnology is also efficient in favor drug tumor accumulation: the nanosized range enables the preferential migration of NPs in the tumor tissue taking advantage of enhanced permeation and retention effect (EPR). Generally, tumor vessels are more porous than normal tissues allowing NPs to move in easily [100]. In this regard, the biodistribution study of RB derivative-chitosan NPs [22] proved that carriers selectively accumulated in tumor lesions, and, in the same way, PGA-RB NPs tumor accumulation increased compared to free RB [76]. Besides, PGA-RB NPs improved the blood circulation time of RB: increasing RB circulatory half-life is currently an open challenge and is a crucial point to improve RB-based therapy. Nanomedicine strategies in this sense typically consist of coating particle surface with ligands that could minimize NPs protein absorption or using PEGylation to avoid opsonization [90,100]. Son et al loaded the photosensitizer drug pheophorbide (Pba) into PLGA NPs and observed that the blood circulation time of formulated Pba was longer than free drug [101]. Besides, NPs below 200 nm-sized showed an enhanced circulation time irrespective of modifying their surface [90].

RB can be considered a multifunctional drug since it can be used both for its intrinsic cytotoxicity and as a photosensitizer drug. Although these two medical properties rely on different principles, in both cases, RB needs to be efficiently internalized by cells selectively, and the need for a longer circulation time persists. In this sense, the abovementioned advantages of formulating RB in nanocarriers remain valid even if nanocarriers are intended for sono-photodynamic treatments. In PDT/SDT context, nanocarriers have demonstrated not to interfere with sono-photochemical treatment; thus, drug release is not strictly required, but it is necessary to ensure that physical-chemical properties of PS are not compromised affecting its phototoxicity [54]. Additionally, in the PDT-SDT context, the aspects of molecular aggregation, photo-sonosensitivity phenomena, and “light therapeutic window” need to be considered.

According to the literature, the association of PS to NPs could help overcome the difficulties related to PS aggregation in the physiological environment preventing the decrease of ROS production. A recent paper by Obinu et al [102] demonstrated that loading indocyanine green (ICG)

into poly (ethyl 2-cyanoacrylate) NPs stabilized the dye and prevented the formation of molecular aggregates, which were observed in free ICG solution. Kohle and collaborators synthesized ultrasmall PEGylated organic-inorganic hybrid silica NPs carrying a methylene blue derivative; NPs can provide targeted delivery and avoided derivative aggregation responsible for the ROS production decrease.[103]. A similar hypothesis may be advanced for RB. Solid peptide NPs loaded with RB in the order of concentration of 10^{-6} M, did not show adhesion to each other demonstrating that encapsulation of RB may prevent aggregation.[104].

Another aspect that should be considered is the premature sono-photodegradation of photosensitizer drugs. To prove that nanocarriers can solve this issue, Gianotti et al [72] compared the UV-Vis spectra of RB solution and RB formulated in mesoporous silica NPs (MSNs) after prolonged exposition to visible light. The decrease of RB absorption band due to photodegradation is evident for free RB and is almost stopped when the dye is associated with MSNs. Hence, nanocarriers can potentially protect RB from photodegradation with the consequence of an improved PDT efficacy. Nevertheless, this technological aspect is not considered by all the RB delivery systems developed, and a more in-depth investigation in this way could help understand which types of NPs are best suited to the purpose.

A limit usually reported for PS is the lack of selectivity for the target tissue resulting in a nonspecific biodistribution throughout the body that may cause photoallergic reactions. In this case, RB shows the advantage of an intrinsic selectivity for cancer and microbial cells, as previously discussed. Wang et al studied the specificity of gold nanorods and RB coated gold nanorods to oral cancer cells, demonstrating that RB coated nanorods were more easily uptaken into the oral cancer cells attributing the outcome to RB intrinsic specificity; furthermore, RB showed higher specificity on cancer cells compared to normal mucosal epithelial cells [21]. The absence of significant phototoxicity in normal skin upon green light irradiation of topical RB has been documented [30]; on the other side, systemic phototoxicity following intralesional RB was reported as well [105]. This point still needs to be made clear, and further investigation is necessary both for free and formulated RB.

As previously reported, RB maximum absorption wavelength is out of the so-called “light therapeutic window” limiting free RB light-mediated treatments to the most superficial skin layers. In this regard, upconversion nanoparticles proved an effective depth-tissue light-guiding ability representing a promising approach to apply RB-PDT therapy even to inner organs [81,82].

6. Conclusion

In the current review, RB therapeutic potential has been discussed, and its strengths and weaknesses from a pharmaceutical point of view have been analyzed. The review aimed to understand if advanced delivery systems could be effective in overcoming RB disadvantages. Through the revision of RB research articles about this topic, it emerges how a proper RB formulation and delivery system increase RB cell uptake and cell accumulation in a selective manner and, consequently, the therapeutic efficiency is improved. Our review highlights how nanotechnological devices are widely employed to overcome RB limits as a therapeutic agent. Considering both RB therapeutic potential and spectrochemical properties, further studies could be carried out on developing delivery systems for the theranostic field, working both as diagnostic imaging instruments and as a therapeutic agent.

7. References

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Chapter II.

Transfersomes: the newly developed Rose Bengal delivery system to target and manage cutaneous and metastatic Melanoma.

Melanoma is a malignancy of melanocytes, the pigment-producing cells of neuroectodermal origin that can be found throughout the body (including in the skin, iris, and rectum). With more than 1.5 million new cases estimated in 2020, skin cancers are the most commonly diagnosed malignancies worldwide [1,2]. According to the Italian Association for Cancer Research (AIRC), malignant melanoma represents only 5% of skin cancers; nevertheless, its ability to rapidly metastasise makes melanoma the most aggressive form, accounting for the majority (75%) of deaths related to skin cancer. Historically a rare disease, melanoma incidence rates have increased worldwide during the last 50 years, especially where fair-skinned people have excessive sun exposure. Indeed, much of this increase is likely due to increased exposure to UV radiation, a strong and ubiquitous risk factor for melanoma, emitted naturally by the sun but also from artificial sources. The rates and number of melanoma diagnoses are projected to increase in the coming decades emphasising the urgent need for targeted melanoma control measures [1,2]. The therapeutic options currently available depend on the stage of the disease, as well as the location of the melanoma tumour.

Cutaneous melanoma can be divided into *in situ* malignant melanoma (ISM) and advanced malignant melanoma (AM). Cutaneous melanoma is classified as *in situ* if confined within the epidermis or advanced when atypical melanocytes progressively infiltrate into the dermis [3]. Tumour thickness (Breslow score), lymph node involvement, and the presence of metastasis define melanoma staging. Melanoma severity is classified into four stages: stage I and II refer to localised disease, and stage III and stage IV are characterised by metastasis to the local lymph nodes or distant metastasis, respectively [4]. Surgical removal is the prevailing treatment option for patients diagnosed with ISM and is often resolute [2]. However, surgical treatment is not curative for AM. In this case, the prognosis is poor after surgical resection since melanoma is the solid tumour with the highest number of mutations, and cancer cells can evade the immune system [4]. To date, non-

surgical therapeutic approaches for AM include chemotherapy, immunotherapy, radiotherapy and photodynamic therapy; however, they are not showing efficacy due to their low ability to accumulate into the tumour tissue or to the lack of specificity [4,5]. As a result, the pharmacological intervention may cause serious side effects related to immune reactions [4]. Therefore, developing a new, more efficient, and safer protocol for melanoma treatment is necessary.

Considering where melanoma resides, particularly at the earliest stages, cancer cells would be easily achievable by the percutaneous administration of therapeutics, with the advantage of avoiding systemic administration and related complications. Indeed, percutaneous delivery offers several benefits, including i) overcoming oral delivery limitations, such as vomiting and low bioavailability due to the first pass metabolism; (ii) representing a replacement for conventional parenteral administration, which can be invasive and painful and risks bruising and infection; (iii) providing a relatively consistent drug concentration in the systemic circulation over a prolonged time. Nevertheless, the skin is a widely documented barrier to external agent penetration. The original “500 Dalton rule” as the maximum molecular weight for skin penetration is still applicable. It is also reliable the general knowledge that hydrosoluble and poor lipophilic active pharmaceutical ingredients (APIs), such as Rose Bengal (RB), rarely overcome the stratum corneum (SC) barrier, resulting in an insignificant topical and transdermal bioavailability and a consequent therapeutic efficiency. A proper drug delivery system (DDS) is mandatory to maximise the pharmacological activity of these APIs.

Vesicular DDS are highly ordered assemblies of one or more concentric phospholipid bilayers enclosing an aqueous core [6]. Liposomes represent the simplest, and the ancestor vesicular DDS developed and were first reported as skin topical DDS in 1980. Since then, investigating vesicular systems applied to the percutaneous route has exponentially increased [7]. The advantages of using vesicular DDS percutaneously, including epidermal, dermal, and transdermal delivery, are based on the possibility of encapsulating both hydrophilic and hydrophobic APIs, increasing their diffusion across the skin. Conventional liposomes may act by four mechanisms [8,9]:

1. The drug-free mechanism, where the API is initially released from the vesicle, diffuses through the skin by an independent permeation;
2. Collision complex transfer between the API intercalated in the bilayer and the outmost surface of SC via a thin hydrophilic film at the interface;
3. Penetration enhancement effect due to ultrastructural changes in the SC when treated with a high concentration of phospholipids;
4. Adhesion onto the skin surface, with adsorption to and/or fusion with the lipid matrix of SC, thereby increasing drug partitioning into the skin.

Nevertheless, the limit with conventional liposomes is that they do not deeply penetrate the viable skin and blood circulation; therefore, they have been extensively applied for epidermal and dermal delivery but not for transdermal absorption [8]. Over the years, the liposome's structure has been submitted to several modifications resulting in the successful formulation of optimised vesicular DDS, namely niosomes, ethosomes, transfersomes and transethosomes [10–13]. Transfersomes (TFs) are known to be the most innovative dermal and transdermal DDS since they have been reported to invade the skin intact and go deep enough to be absorbed by systemic circulation [8]. TF are vesicular DDS with the additional presence of surfactants (edge activators, EAs) in their composition; the typical surfactant concentration used and recommended is between 10 and 25%, and the phospholipid: EA ratio of 85:15 is frequently reported when nonionic surfactants are used [7]. Because of their composition, the lipid bilayer fluidity increases and lead to the ultra-deformability of TF, which is the property to temporarily change their shape when in proximity to the skin's pore and migrating through them. The driving force for the migration of TF is likely to be the water concentration gradient that develops across the skin, the so-called "transdermal hydration gradient". Phospholipid hydrophilicity causes the vesicles to resist dehydration at the skin surface, moving into deeper and water-richer skin strata. This force is insufficient for conventional liposomes; indeed, liposomes are defined by a rigid structure that hampers their migration to the deepest skin strata as they require high energy costs to deform, resulting in breaking and drug leaking. Conversely, the EA molecules in TF tend to accumulate at the site of high stress due to their propensity for significantly curved structures reducing the energy required for deformation, which, being flexible, can follow the transdermal hydration gradient reaching blood and lymph capillaries intact [7,9].

Finally, TF represents an innovative DDS to manage skin-related disorders, including skin cancers. This is evidenced by the fact that several investigations have been carried out proposing the use of TF or their successors (ethosomes, transethosomes, etc.) for skin cancer therapy (table 1).

Table 1. Deformable Vesicular DDS reported in the literature from 2018 to 2022 for targeted skin cancer therapy. API: Active Pharmaceutical Ingredient.

<i>Vesicular DDS</i>	<i>Skin Cancer</i>	<i>API</i>	<i>Ref.</i>
Transethosomes	Melanoma	Fe-chlorophyllin	[14]
Transethosomes	epidermoid carcinoma	epigallocatechin-3-gallate	[15]

Ethosomes Deformable DDS			
Transfersomes	UV-induced skin cancer	Carvedilol	[16]
Ethosomes Transfersomes	Melanoma	Sulphorane	[17]
Deformable DDS	epidermoid carcinoma	Nobiletin	[18]
Deformable DDS	Basal cell carcinoma	Vismodegib	[19]
Transfersomes	Melanoma	Paclitaxel	[20]

Based on what was stated above, section I of the current Thesis chapter reports the development and characterisation of Rose Bengal loaded TF (RBTF) for *in situ* melanoma therapy [21]. The investigation concluded that the newly developed formulation significantly improved RB permeation through the epidermis. Moreover, melanoma cell growth significantly decreased when RB was associated with TF, and the selectivity towards cancer cells increased compared to the free drug. Nevertheless, aiming to treat AM that infiltrates more profoundly into the skin, the topical application of the RBTF dispersion could limit the therapeutic advantages of RBTF vesicles. The epidermis permeation test proved that about 20% of the drug couldn't reach the dermis remaining intercalated within the outmost epidermal layers. As previously reported, the ultra-deformability of TF is supposed to be the primary operating mechanism for dermal and transdermal delivery. Still, the mechanisms typically ascribed to conventional liposomes cannot be excluded. It's likely that part of the RBTF either lost their main structural characteristics during the migration through the epidermis or leaked, causing undesired drug waste and reducing the RB amount deposited in the dermis.

Set the sight on advanced melanoma management, the second step of the PhD project focused on including RBTF into a dosage form that could maximise their deposition in deepest skin strata while maintaining the integrity of their structure and the drug release rate. For this purpose, microneedle technology was considered.

Microneedles (MNs) have been widely investigated for delivering APIs through the percutaneous route overcoming the limitations of the standard approaches. Considering the discomfort caused by hypodermic needles and the weak points of transdermal patches, MNs were developed to be a

hybrid of both. They represent a microinjection device that provides a rapid increase of local APIs concentration and, consequently, enhances the dermis deposition and the systemic drug absorption of several active agents, including hydrosoluble, hydrophilic, and high molecular weights molecules, biologics, and nanoparticles (NP) [22–24]. MNs array consists of a small patch carrying a multitude of thin micron-sized needles (25–1500 μm), which bypass the SC by creating reversible transient microchannels in the skin [23,25]. Remarkably, the needles are long enough to overcome the SC barrier but short enough to be painless. Clinical applications demonstrated that MNs do not stimulate nerves associated with pain and do not cause infections or irritation, resulting in improved patient compliance. Furthermore, MNs are self-administrable and do not generate medical sharps waste [24]. Using NPs in combination with MNs would allow NPs to be directly deposited into the deeper skin strata triggering a multimodal synergistic activity of both drug delivery technologies, finally maximising the therapeutic potential of their cargo [25]. Five different types of MNs have been reported in the literature: solid, coated, hollow, polymeric dissolving, and hydrogel-forming MNs. The current thesis describes the use of polymeric dissolving MNs (DMNs) and hollow MNs (HMNs) to target AM disease.

DMNs are fabricated with hydrosoluble, biodegradable, and biocompatible polymers, which dissolve in the skin tissue and slowly release the NPs or the drug encapsulated in the NPs. The delivery mechanism is “poke and dissolve” (figure 2). The application requires only one step since the polymer degrades inside the skin, and no post-treatment removal is necessary. By regulating the dissolution rate of the polymeric matrix, a controlled and sustained NP delivery can be achieved [25]. In the specific case of lipid NPs, the primary limit of DMNs relies on the relatively poor drug loading due to the dual challenge of providing tips strong enough to perforate the skin while loading in their matrix-softening materials, such as phospholipids and surfactants [26,27]. This was confirmed in the case of RBTF (Section II, Chapter II) and limited the RBTF-loaded DMNs application to AM at stages I and II [28]. Viewing the shortcomings of RBTF-loaded DMNs, the technology of HMNs was thought to better suits the management of stage III and IV of AM, which are defined by the metastatisation process, in which the lymphatic system plays a crucial role. Therefore, as the last step of this project, the RBTF lymphatic delivery mediated by HMNs was investigated. Targeted delivery of therapeutic to lymphatic vessels and nodes, also known as lymphotropic delivery, is necessary for treating and preventing diseases with central lymphatic involvement, including cancer. The intradermal space achieved by MNs is rich in lymph capillaries and represents an easy way to access the lymphatic system. HMNs act through the “poke and flow” approach and are represented by one or multiple hypodermic needles of micron-sized in length and diameter

connected to a source of the injectable formulation. The essential benefit is that it allows the dose to be easily controlled based on patients' needs while reporting higher compliance than the hypodermic injection [26,29].

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Research Article

Improving Dermal Delivery of Rose Bengal by Deformable Lipid Nanovesicles for Topical Treatment of Melanoma

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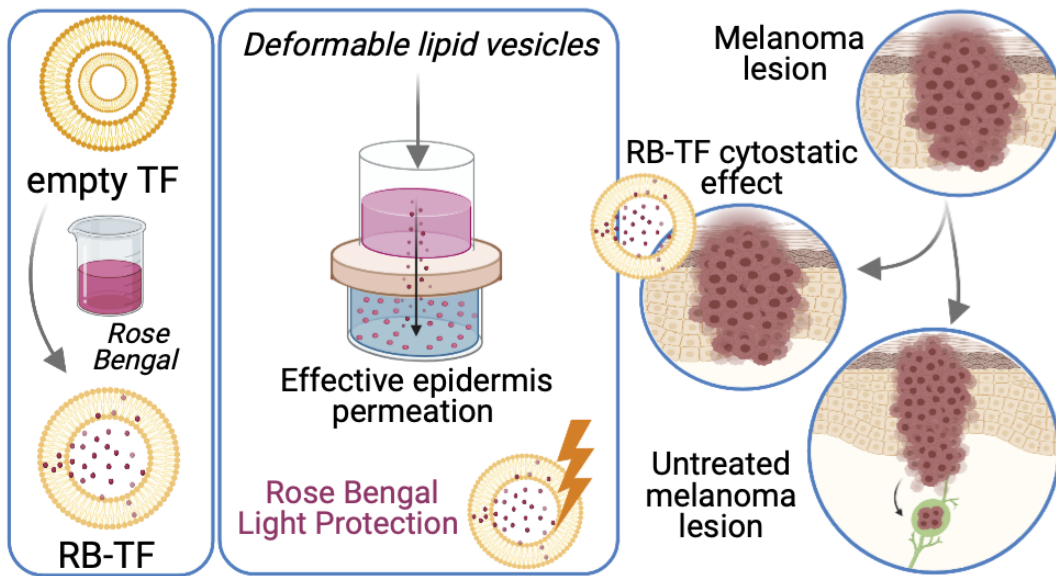
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Herein, the first experimental section of the PhD project is presented, focused on the formulation and physicochemical and biological characterization of the Rose Bengal loaded Transfersomes carried out in the first academic year and a half of the PhD. The investigation was finally published as an Original Research Article in the peer-reviewed and indexed journal “*Molecular Pharmaceutics*”; (2021 Journal Impact Factor (JIF): 5.364; JIF Quartile: Q1 in the category Pharmacology and Pharmacy, Q2 in the category Medicine, Research & Experimental).

Abstract

Cutaneous melanoma is one of the most aggressive and metastatic forms of skin cancer. However, current therapeutic options present several limitations, and the annual death rate due to melanoma increases every year. Dermal delivery of nanomedicines can effectively eradicate primary melanoma lesions, avoid the metastatic process, and improve survival. Rose Bengal (RB) is a sono-photosensitizer drug with intrinsic cytotoxicity towards melanoma without external stimuli, but the biopharmaceutical profile limits its clinical use. Here, we propose deformable lipid nanovesicles, also known as transfersomes (TF), for the targeted dermal delivery of RB to melanoma lesions to eradicate them in the absence of external stimuli. Considering RB's poor ability to cross the stratum corneum and its photosensitizer nature, transfersomal carriers were selected simultaneously to enhance RB penetration to the deepest skin layers and protect RB from undesired photodegradation. RB loaded TF dispersion (RB-TF), prepared by a modified reverse-phase evaporation method, were nanosized with a zeta potential value below -30 mV. The spectrophotometric and fluorometric analysis revealed that RB efficiently interacted with the lipid phase. The morphological investigations (TEM and small-angle x-ray scattering) proved that RB intercalated within the phospholipid bilayer of TF originating unilamellar and deformable vesicles, in contrast to the rigid multilamellar unloaded ones. Such outcomes agree with the results of the *in vitro* permeation study, where the lack of a burst RB permeation peak for RB-TF, observed instead for the free drug, suggests that a significant amount of RB interacted with lipid nanovesicles. Also, RB-TF proved to protect RB from undesired photodegradation over 24 hours of direct light exposure. The *ex vivo* epidermis permeation study proved that RB-TF significantly increased RB's amount permeating the epidermis compared to the free drug (78.31% vs. 38.31%). Finally, the antiproliferative assays on melanoma cells suggested that RB-TF effectively reduced cell growth compared to free RB at the concentrations tested (25 and 50 μ M). RB-TF could potentially increase selectivity towards cancer cells. Considering the outcomes of the characterization and cytotoxicity studies performed on RB-TF, we conclude that RB-TF represents a valid potential alternative tool to fight against primary melanoma lesions via dermal delivery in the absence of light.

Keywords: Liposome; Nanoparticle; Rose Bengal; Melanoma; Skin cancer; Dermal delivery.



1. Introduction.

According to the Italian Association for Cancer Research (AIRC), cutaneous melanoma represents only 5% of skin cancers. Nevertheless, its ability to rapidly metastasize makes melanoma the most aggressive and deadly form of skin cancer. It remains a significant health problem worldwide, with death often occurring due to metastasis [1]. Statistics predicted that around 207,390 cases of melanoma would be diagnosed in the United States in 2021, and death is expected to increase by 4.8% [2]; also, the incidence of invasive melanoma in European regions showed an average annual increase of 4.0 % in men and 3.0% in women in 1995-2012 period [3]. Cutaneous melanoma typically localizes in the epidermis' bottom layers or deeper in the dermis and originates from melanocytes' tumor transformation. Depending on the location and stage, the primary cutaneous melanoma can be treated via surgical resection, but it cannot be applied in all cases. Current therapeutic approaches, including chemotherapy, radiotherapy, immunotherapy, and photodynamic therapy, have reported two main limitations: adverse effects (usually related to an immune reaction and lack of specificity) and induced resistance to immune-chemotherapeutics or intralesional therapy [4]. Thus, better therapeutic options are required. For this purpose, primary melanoma lesions can be effectively eradicated via dermal delivery of therapeutics, increasing patient compliance. Indeed, the dermal route offers direct access to melanoma cells avoiding systemic administration and its disadvantages. Considering the complexity of human skin, the challenge in developing an effective dermal delivery system is to bypass the skin barrier and target the drug only to cancer cells[1,5].

Rose Bengal (RB) (3', 4', 5', 6'-tetrachloro-2,4,5,7-tetraiodofluorescein) is a dye with a xanthenic structure mainly used in ophthalmology as a diagnostic tool [6] (figure 1).

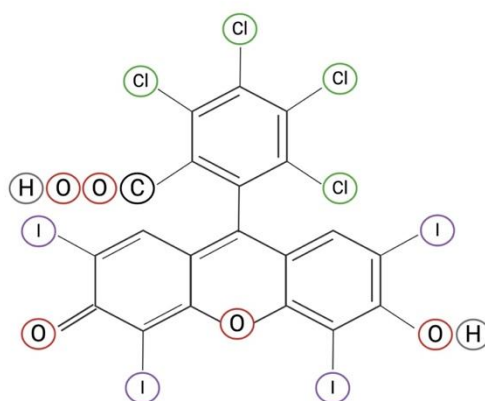


Fig. 1. *Rose Bengal chemical structure (created with BioRender.com).*

Moreover, RB is a sono-photosensitizer drug employed in sono-photodynamic therapy and demonstrated intrinsic cytotoxicity against tumour and microbial cells [6]. Of relevant importance in this context is RB's anti-melanoma activity in the absence of external stimuli, including light or ultrasounds. In particular, RB was reported to induce death in different cultured melanoma cell lines, probably via necrosis, even if both caspase-dependent and independent apoptotic pathways were observed [7,8]. PV-10® is a 10% RB solution currently tested to treat melanoma after intralesional injection. PV-10® acts through a dual mechanism: it first induces a chemical ablation of the tumour and a second systemic effect following the onset of a tumour-specific immune response[9,10]. Therefore, RB can be proposed as a valid alternative for both local and metastatic melanoma.

Despite its potential, RB's clinical application is limited by its biopharmaceutical profile. RB (employed in the disodium salt form) is an amphiphilic and water-soluble molecule of 1017.64 g/mol, presenting two negative charges in solution [6]. For an efficient permeation through the skin layers, a drug first needs to pass the stratum corneum (SC), which is the main barrier against the penetration of external agents. Considering the anionic nature and the high molecular weight, RB would not easily pass the SC and therefore cannot be considered a drug candidate suitable for the cutaneous administration route [11,12]. Besides, RB suffers from poor cell crossing hampering the proper accumulation in the target cells. Nevertheless, nano-based delivery systems have proved to overcome RB limits mentioned above efficiently [6].

Lipid-based nanosystems were widely employed for the dermal delivery of photosensitizer drugs [13,14]. Among these, transfersomes (TF) are considered the most innovative dermal and transdermal carrier to this date [15]. TF are liposome-like vesicles consisting of a hydrophilic core surrounded by a hydrophobic bilayer, but the additional surfactant presence in the phospholipid arrangement provides an ultra-flexibility [16]. A conventional liposome presents the disadvantage of poor penetration into the viable skin, limiting its therapeutical application to the diseases involving outmost skin layers. Because of its composition, the elasticity of TF increases, making them squeeze and pass-through skin pores smaller than their size. Considering that TF tend to avoid dry surroundings, the sufficient force to drive them through the skin is generated by the transdermal hydration gradient, the increasing water concentration gradient from the superficial to the inner skin strata. In conventional liposomes, the rigid structure hampers the migration as they are

required of high energy cost to deform. On the other side, TF can deform without losing their integrity, and undesirable leakage and drug loss are avoided. As a result, the drug is efficiently delivered in the deepest region of the skin [17,18]. Although the mechanism above is considered the primary operating, other mechanisms can be involved in the transfersomal action [18,19].

The current work aims to provide a dermal delivery of RB to melanoma lesions via optimizing its biopharmaceutical profile and enhancing RB's intrinsic anti-melanoma activity in the absence of light or ultrasounds. To achieve this goal, RB has been formulated in TF made of phosphatidylcholine (Lipoid S100), cholesterol and Span®80. RB-loaded TF were characterized in dimensional properties, zeta potential and morphology; besides, the storage stability and photostability of RB-loaded TF and corresponding blank formulation were determined. Spectrophotometric, fluorometric, and small-angle X-ray scattering (SAXS) analyses were performed to evaluate the interaction between the dye and the lipid phase of vesicles. *Ex vivo* epidermis permeation experiment determined TF's ability to permeate across the epidermal barrier reaching the dermis. Finally, an *in vitro* toxicity study on melanoma cells (SK-MEL28) was carried out to investigate the new RB delivery system's anti-melanoma efficacy.

2. Materials and methods

2.1 Materials.

Rose Bengal sodium salt (RB), cholesterol, Span® 80, and ethanol were purchased by Sigma-Aldrich (St. Louis, MO). Lipoid S 100 was gifted by Lipoid GmbH (Ludwigshafen, Germany). Acetonitrile and dimethyl sulfoxide (DMSO) were acquired from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS, NaCl 0.138 M; KCl 0.0027 M; pH 7.4; 25 °C) was obtained by Sigma-Aldrich (Milan, Italy). Ultrapure bi-distilled water was obtained by a MilliQ R4 system, Millipore (Milan, Italy).

2.2 Preparation and characterization of TF

2.2.1 TF Preparation.

A previous preformulation study set the most suitable manufacturing parameters to prepare unloaded and RB-loaded TF (Supporting Information), including the preparative technique, the choice of the organic solvent, and the sonication technique and time.

RB-loaded TF dispersion (RB-TF) were prepared by a slightly modified reverse phase evaporation method (REV) [20]. The lipid phase consisting of a mixture of 400 mg of Lipoid S100 (phosphatidylcholine), 75 mg of cholesterol, and 40 μ L of Span[®] 80 was dissolved in 7 mL of ethanol at 50 °C and mixed with 10 mL of an aqueous solution (MilliQ water) containing 5 mg of RB. After sonication treatment by probe sonicator Bioblock Vibracell (Fisher Bioblock Scientific, Illkirch, France) for 30 s at 50% ultrasound (US) amplitude, the resulting dispersion was placed in a dry, round bottom flask. Ethanol was removed under vacuum (Rotavapor RE111, Büchi Labortechnik AG, Flawil, Switzerland) at 50 °C, and the vesicles were swollen for 1 h at room temperature and sonicated by three probe sonication cycles: each cycle consisted of 10 s of US followed by an interval of 20 s[21]. Finally, RB-TF dispersion was extruded 10 times through a regenerated cellulose syringe filter (pore size: 0.45 μ m, filter size: 25 mm, AlfaTech, Genova, Italy) and stored at 4°C. Corresponding unloaded vesicles dispersion (b-TF) were prepared.

2.2.2 Particle size and zeta potential analysis.

Dimensional analyses of RB-TF and b-TF were performed by photon correlation spectroscopy (PCS). A Coulter nanosizer N5 (Beckman-Coulter Inc., Miami, FL, USA) was used to determine the hydrodynamic diameter and the polydispersity index (PDI) to measure the dimensional heterogeneity of the sample. Before each analysis, all samples were diluted adequately with MilliQ water previously filtered (regenerated cellulose syringe filter, pore size: 0.20 μ m, filter size: 15mm, Albet LabScience, Dassel, Germany) to ensure light-scattering intensity within the required range of the instrument (between 5×10^4 and 1×10^6 counts s^{-1}). Particle size was calculated in unimodal using the following conditions: fluid refractive index 1.333; temperature 25°C; viscosity 0.890 centipoises; angle of measurement 90°; sample time 3.0 ms and sample run time 300 s. Zeta potential was measured using the Zetasizer Nano (Malvern Instrument, Worcestershire, United Kingdom) through the M3-PALS (Phase Analysis Light Scattering) technique. Just before the analysis, the formulations were diluted with distilled water.

2.2.3 Drug content.

RB content in the RB-TF dispersion was evaluated as described here. Thirty μ L of RB-TF dispersion were diluted up to 2 mL with acetonitrile under magnetic stirring to dissolve the nanovesicles and extract RB. Successively, RB content was measured by UV-spectrophotometer (SHIMADZU UV-1800, Kyoto, Japan) and calculated referring to the calibration curve prepared in

acetonitrile (standard solution in the range of 1-10 mg L⁻¹; $y=101610959x+0.020676712$; $R^2=0.999$).

Finally, the drug content (DC%) was determined according to equation (1):

$$DC\% = \frac{\text{Amount of RB in RB - TF dispersion}}{\text{Theoretical amount of RB used}} \times 100 \quad (1)$$

2.2.4 Transmission Electron Microscopy (TEM).

RB-TF and b-TF were analyzed through transmission electron microscopy (TEM) to investigate the vesicles' morphology. A drop of sample and an equal volume of an aqueous 1% phosphotungstic acid solution were adsorbed on the surface of a carbon-coated copper grid (200 mesh). The vesicles were observed after drying at room temperature using a JEOL JEM 1400 Plus (JEOL Ltd., Tokyo, Japan) with an accelerating voltage of 80 kV in Bright-field mode.

2.2.5 Degree of deformability.

Deformability tests of b-TF and RB-TF were performed using a modified extrusion device [22] illustrated in figure 2.

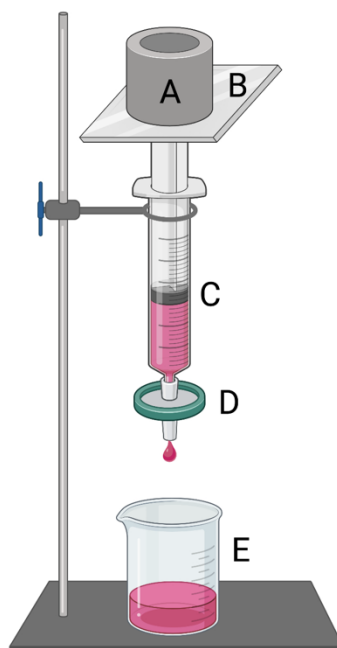


Fig. 2. Schematic illustration of the modified extrusion device employed to evaluate the deformability index of RB-TF and b-TF. A: 500 g calibration weight; B: weight support; C: syringe; D: support for the membrane filter (0.2 μm pore size); E: collecting beaker. Created with BioRender.com.

Sample (2 mL) were withdrawn with a syringe successively connected to a membrane filter with a pore size of 0.2 μm (regenerated cellulose membrane filter, filter size: 47 mm, Whatman®, GE

Healthcare Life Sciences, Buckinghamshire, UK) [23,24]; extrusion process was performed by constantly applying a calibration weight of 500 g on the top of the syringe. Particle size was determined just before and after experimenting; deformability index was measured following the equation [25] (2):

$$D = J \left(\frac{r_v}{r_p} \right)^2 \quad (2)$$

Where D is the deformability index of nanovesicles; J is the amount of formulation extruded in 5 min; r_v is the particle size after the experiment; r_p is the membrane pore size.

2.2.6 Small-angle X-ray scattering (SAXS).

SAXS was recorded with an S3-MICRO SWAXS camera system (HECUS X-ray Systems, Graz, Austria). A GeniX X-ray generator, operating at 1mA and 50 kV, provided a Cu K α radiation of 1.542 Å wavelength. The scattered X-rays were detected in the small-angle region by a 1D-PSD-50 M system (HECUS X-ray Systems, Graz, Austria) containing 1024 channels of 54.0 mm width. The working q -range (Å) was $0.002 \leq q \leq 0.06$, with $q = 4\pi \sin(\theta)\lambda^{-1}$ being the modulus of the scattering wave vector. 2 mm glass capillaries were used in experiments while diffraction patterns were recorded for 10800 s. Scattering from the air was minimized, keeping the camera volume under vacuum during measurements. Silver behenate (CH₃-(CH₂)₂₀-COOAg), with a d spacing value of 58.38 Å was the standard for calibration of the angular scale of the measured intensity. SAXS pattern of RB-TF was analyzed by GAP (Global Analysis Program), which allows fitting of bilayer-based structures [19,26]. Particularly, the membrane thickness (d_B) was defined as $2(Z_H + 2\sigma_H)$, where Z_H and σ_H are parameters obtained from SAXS curve fitting and respectively represent the head group-to-bilayer center distance and the polar head amplitude. The latter parameter was kept fixed at 3 Å as suggested by the software developers [26].

2.3 Evaluation of the interaction between RB and lipid phase.

Studies on absorption (Abs) and emission (Ems) spectra of i) RB aqueous solution, ii) RB-TF dispersion, and iii) RB added in b-TF dispersion were recorded, as previously proposed by Chang et al. [27]. Abs spectra were measured with UV-Spectrophotometer (SHIMADZU UV-1800, Kyoto, Japan); Ems spectra were recorded with an RF-600 spectrofluorometer (Shimadzu, Kyoto, Japan), exciting each sample at 549 nm. Before each measurement, all samples were diluted with MilliQ water previously filtered (regenerated cellulose syringe filter, pore size: 0.20 µm, filter size: 15mm,

Albet LabScience, Dassel, Germany) up to 7.5 mg L⁻¹ RB concentration. Spectra of b-TF dispersion were recorded following the same procedure.

2.4 Stability studies

2.4.1 Storage stability.

b-TF and RB-TF dispersions were stored for 60 days at 4°C, protected from light. At each predetermined time point (1, 3, 7, 15, 30, 60 days), samples were analyzed in terms of dimensional properties and zeta potential as described in section 2.2.2 *Particle size and zeta potential analysis*.

2.4.2 Chemical stability.

The chemical stability of RB-TF dispersion was evaluated in terms of RB content over time and compared to RB aqueous solution after storing samples at 4°C protected from light. Samples were analyzed at each predetermined time point (1, 3, 7, 15, 30, 60 days), as described in section 2.2.3 *Drug content*. The RB amount was calculated for the RB aqueous solution, referring to the calibration curve prepared in water (standard solution in the range of 1-20 mg L⁻¹; $y=0.10640119x+0.00531086$; $R^2=0.999$).

2.4.3 Photostability.

The photostability of RB-TF dispersion was evaluated and compared to RB aqueous solution. One mL of each sample (0.5 mg of RB) was exposed to light in the visible region delivered from an artificial daylight lamp (220 Volt, 40 Watt) over 24 h at room temperature. Photodegradation of RB was monitored at each predetermined time point (1 h, 2 h, 3 h, 6 h, 24 h) in terms of RB content by UV-spectrophotometry. RB content in RB-TF dispersion was determined as described in section 2.2.3 *Drug content*; for RB aqueous solution, the amount of RB was calculated referring to the calibration curve prepared in water previously mentioned.

2.5 *In vitro* release study.

In vitro release profile of RB-TF dispersion was evaluated across a polycarbonate membrane (0.050 µm pore size, 47 mm diameter, Sigma-Aldrich, St. Louis, MO, USA) using a dissolution apparatus (DT 70, Erweka, Langen, Germany), as previously described by Gavini et al. [28]. The membrane was mounted on the bottom of cylindrical plastic support consisting of a tube (height=1.91 cm, diameter=2.28 cm) connected to a drive shaft of the dissolution apparatus (Supporting Information). The membrane was clamped to the support by a plastic ring, and then 2

mL of the sample (1 mg of RB) were placed on the surface of the membrane. The system was then inserted into the vessel containing 250 mL of PBS as the acceptor medium, keeping the membrane in contact with the surface of the PBS all the time. The system was continuously stirred at 100 rpm and thermostated at 32°C; it was allowed to equilibrate for 30 min before starting the experiment. Samples of acceptor medium (3 mL) were collected at each predetermined time point (0.08 h, 0.17 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 6 h, 24 h, 32 h, 48 h); in case of RB-TF dispersion, acetonitrile was used to assure the complete recovery of RB. RB aqueous solution was also tested as comparison. The amount of dye in the acceptor medium was measured by UV-Vis spectrophotometry and calculated using the calibration curve prepared in acetonitrile for RB-TF dispersion and in water for RB. An equal volume of fresh medium was immediately replaced after each sampling. The cumulative amount of RB was plotted against time and determined through the equation (3):

$$Q_n = C_n \times V_0 + \sum_{i=1}^{n-1} C_i \times V_i \quad (3)$$

Where Q_n is the cumulative amount of drug; C_n is the drug concentration of the acceptor medium at each time point; V_0 is the acceptor medium's volume; C_i is the drug concentration of the sample at each time point, and V_i is the volume of the sample collected at each time point.

2.6 *Ex vivo* epidermis permeation and retention study.

Ex vivo permeations of RB-TF dispersion and RB aqueous solution were performed across epidermal membranes excised from porcine ears. Adult pig ears were obtained from a local slaughterhouse (Milia S.r.L, Approval Number CE IT 1856 M (Regulation EC 853/2004)). Epidermal membranes, comprising viable epidermis and stratum corneum, were prepared by heat separation technique here described [29]: hairs of ears were removed using a hair clipper; shaved ears were immersed in water at 60°C for about 2 minutes; following removal from the water, the epidermis was gently peeled off. The experiment was performed using a 12-multi-well cell culture plates suitability modified [30] (project INCREASE SARDINIA 2016-17, protocol number 31351, University of Sassari).

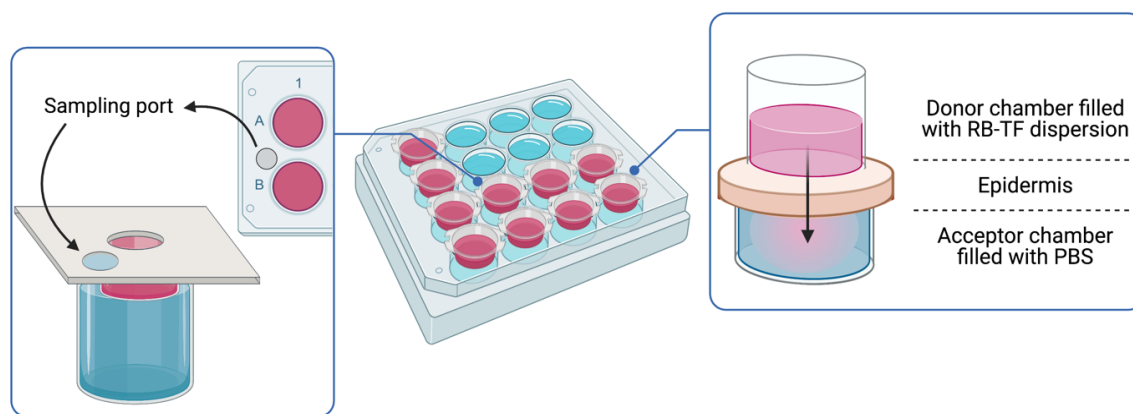


Fig. 3. Schematic illustration of the diffusion apparatus (project INCREASE SARDINIA 2016-17, protocol number 31351, University of Sassari) that was employed to evaluate the *ex vivo* epidermis permeation profile of RB-TF dispersion and RB aqueous solution. Created with BioRender.com.

Each well had an effective diffusion area of 1 cm². Wells were filled with 5.5 mL of PBS (pH. 7.4) as acceptor medium, and the epidermis was mounted on the plates with the inner side facing PBS. 0.3 mL of RB-TF dispersion (0.15 mg of RB) were added on the top of the epidermis, and the plates were incubated at 32°C (SKI 4 Shaker Incubator, ARGO LAB, Carpi, Italy). The system was allowed to equilibrate for 30 min before starting the experiment. Samples (0.2 mL) were withdrawn at each predetermined time point (0.25 h, 0.5 h, 1 h, 3 h, 5 h, 8 h, 24 h) from a sampling port directly connected to the receptor chamber; successively, the samples were added to 1 mL of DMSO to dissolve both lipids of RB-TF dispersion and skin to extract RB completely. The amount of RB permeated was measured by UV-Vis spectrophotometry and calculated using the calibration curve prepared in DMSO (standard solution in the range of 2-12 mg L⁻¹; $y=87.814x-0.1041$, $R^2=0.998$). After each sampling, an equal volume of fresh medium was immediately replaced through the sampling port previously employed to withdraw the sample. The same procedure was applied to evaluate the *ex vivo* permeation profile of the RB aqueous solution. The cumulative amount of RB permeated was plotted against time and determined as described in section 2.5 *In vitro release study*.

At the end of the permeation experiment, the epidermis was carefully removed from the plate and cut into small pieces, added to 2 mL PBS and boiled for 10 min; after that, 2 mL of DMSO were added to assure RB extraction and further boiled for 10 min. The amount of RB retained in the tissue was measured by UV-Vis spectrophotometry and calculated using the DMSO's calibration curve.

2.7 Cytotoxicity and antiproliferative studies.

The antiproliferative and cytotoxic activity of b-TF, RB-TF, and RB aqueous solution at different concentrations (12.5 µM to 50 µM) was evaluated on SK-MEL28 melanoma cells; HFFF2 human fetal

foreskin cell line was tested as a control on normal cells. Cells were cultured in DMEM (Sigma D5671) supplemented with 10% of fetal bovine serum (Euroclone ECS0180L), 2 mM L-Glutamine (VWR X0550), 1 mM Sodium Pyruvate (Sigma S8636), non-essential amino acids (Sigma M7145), 100 units/mL penicillin, 10 µg/mL streptomycin, 25 ng/mL amphotericin B (AA, VWR L0010) at 37 °C with 5% of CO₂. Two thousand cells per well were plated in 384-well microplates and incubated for 24 h at 37 °C with 5% CO₂; the day after, 5 µL of each formulation was added to samples. After 48 hours of treatment, cell death was estimated by measuring green fluorescence intensity, provided by staining dead cells with CellTox Green dye (Promega), and cell viability was evaluated by CellTiter-Glo kit (Promega), following manufacturer's instructions. A second plate was prepared in parallel to assess cell viability at the time of treatment.

2.8 Statistical analysis.

Statistical analysis was done with Graph Pad Prism 8.0 software (GraphPad Software Inc. San Diego, CA, USA). The variance analysis (one-way ANOVA) was used to analyze the data, followed by Tukey's multiple comparison test. Differences were considered significant for $p < 0.05$.

3. Results

3.1 Preparation and characterization of TF.

TF dispersion was milky, white-coloured in the case of b-TF, and pink-coloured for RB-TF dispersion; no phase separation or aggregation phenomena were observed. The particle size and PDI (Table 1) indicate that b-TF were in a nanosized range and homogenous ($PDI < 0.4$); also, b-TF were spherical and multilamellar as seen through TEM (Figure 4). RB-TF showed a PDI similar to b-TF one ($p > 0.05$), proving that encapsulating RB did not perturb the system's dimensional homogeneity. On the other side, a slight increase in particle size was observed compared to b-TF ($p < 0.01$). Besides, nanovesicles surface charge expressed as zeta potential measurements decreased from a slightly negative value to a highly negative one ($p < 0.0001$) following RB loading (Table 1). TEM images (figure 4) revealed a morphological difference between the two formulations, as b-TF were multilamellar vesicles (MLV) whereas RB-TF were spherical large unilamellar vesicles (LUV). Also, the SAXS experiments evidenced the differences in terms of lamellarity between the two formulations. Indeed, the diffractogram related to b-TF (figure 5) revealed the presence of two strong Bragg reflections superimposed to a barely visible diffusive scattering. While the latter was

expected when nanoparticles dispersion was analyzed, the observed Bragg peaks proved, beyond any doubt, the high lamellarity of the vesicles. The average interlayer distance (d_{av}) of 6 nm was calculated from the Bragg relation $d_{av} = 2\pi h/q$, where h is the Miller index and q is the position of the Bragg peaks. On the other hand, the SAXS profile of RB-TF was characterized by a simple diffusive scattering, indicating the absence of lamellarity. To get deeper insights into the transfersome nanostructure, the liposome double layer thickness (d_B) was measure in both b-TF and RB-TF. In the case of b-TF, d_B (indicated by arrows in Figure 4A) was measured exploiting the TEM images using the ImageJ program, and it was found equal to 50 Å. Unfortunately, the same analysis cannot be performed on RB-TF due to the low resolution (see figure 4B). Therefore, in that case, d_B was extracted by fitting the SAXS diffractogram using the Global Analysis Program (GAP, see the Material and Method section). The GAP analysis yielded $Z_H = 18$ Å. Therefore, a double layer thickness of 48 Å was calculated, in good agreement with that measured in b-TF liposomes and other similar nanoparticles reported in the literature [31]. This finding evidenced that the addition of RB to the formulation did not significantly modify the transfersome double layer thickness, although an evolution from MLV to LUV was observed.

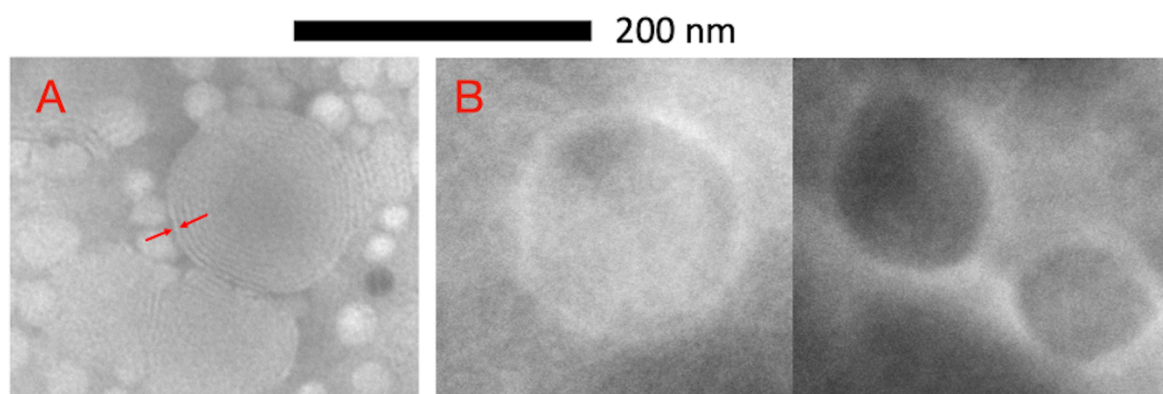


Fig. 4. TEM images of b-TF (A) and RB-TF (B). Scale bar: 200 nm, magnification X30k (A) and X40k (B). Red arrows show the thickness of the lipid double layer (A).

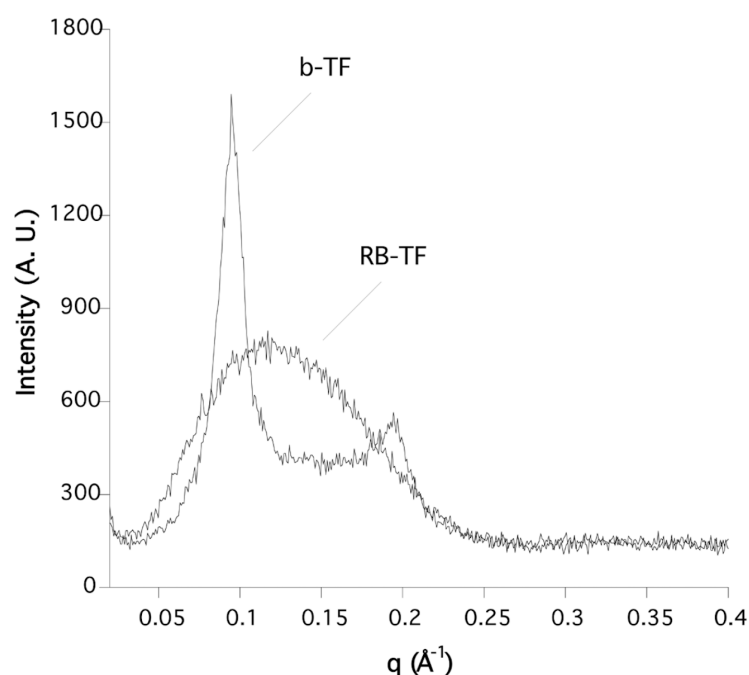


Fig. 5. SAXS diffractograms of *b*-TF and RB-TF.

Particle size and PDI of RB-TF did not change after the deformability test ($p > 0.05$) (size: 195.70 ± 0.95 vs. 196.07 ± 0.31 ; PDI: 0.375 ± 0.002 vs. 0.387 ± 0.011), whereas the size of *b*-TF significantly decreased, revealing no deformable vesicles ($p < 0.01$) (size: 226.67 ± 2.80 vs. 217.20 ± 2.51 ; PDI: 0.215 ± 0.012 vs. 0.239 ± 0.016). Although the membrane pore size employed in the deformability test did not vary much with the particle size of the tested formulation, RB-TF revealed elastic properties since no change in dimensional profile was experienced, contrary to the unloaded formulation.

Table 1. Characteristics of *b*-TF and RB-TF. Results are expressed as mean value $\pm$ standard deviation ($n=6$); Deformability index $n=3$. ** p value < 0.01 vs. RB-TF; *** p value < 0.0001 vs. RB-TF.

	Particle Size (nm)	PDI	Zeta potential (mV)	Loading Efficiency (%)	Deformability Index
<i>b</i>-TF	$193.10 \pm 7.5^{**}$	0.175 ± 0.004	$4.9 \pm 0.9^{***}$	-	Not def.
<i>RB</i>-TF	206.42 ± 2.67	0.200 ± 0.024	45.90 ± 0.85	94.50 ± 3.0	1.92 ± 0.06

3.2 Evaluation of the interaction between RB and lipid phase.

Two analysis techniques evaluated RB's ability to interact with lipid nanovesicles, including spectrophotometry and fluorimetry. Figure 6 reports Abs and Ems spectra of RB in the 450-700 nm range in distinct molecular environments.

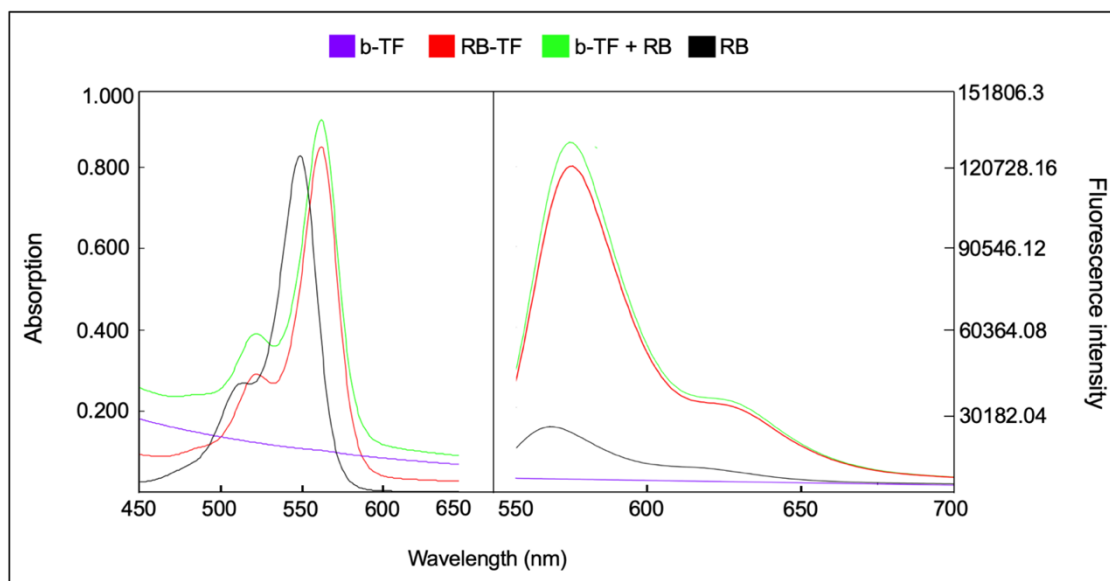


Fig. 6. Absorption and emission spectra of RB (7.5 mg L⁻¹) included in different molecular environments. RB aqueous solution (black line), b-TF (purple line), RB added to b-TF (green line), RB-TF (red line).

The spectra demonstrated that RB Abs and Ems λ_{max} are related to the composition of the environment: 549 nm (Abs) and 568 nm (Ems) for RB aqueous solution; 562 (Abs) and 582 (Ems) for both RB added to b-TF and RB-TF; no peak in Abs or Ems was recorded for b-TF. Both RB Abs λ_{max} and Ems λ_{max} exhibited a shift to a longer wavelength (redshift or bathochromic) when forced to interact with TF. The Abs spectra displayed a slight increase in the RB maximum absorption peak intensity depending on the formulation. In Ems' case, this increase is evident comparing RB aqueous solution to RB-TF and RB added to b-TF.

3.3 Stability study.

The diameter and PDI of TF following storage at 4°C over 60 days are illustrated in Figure 7.

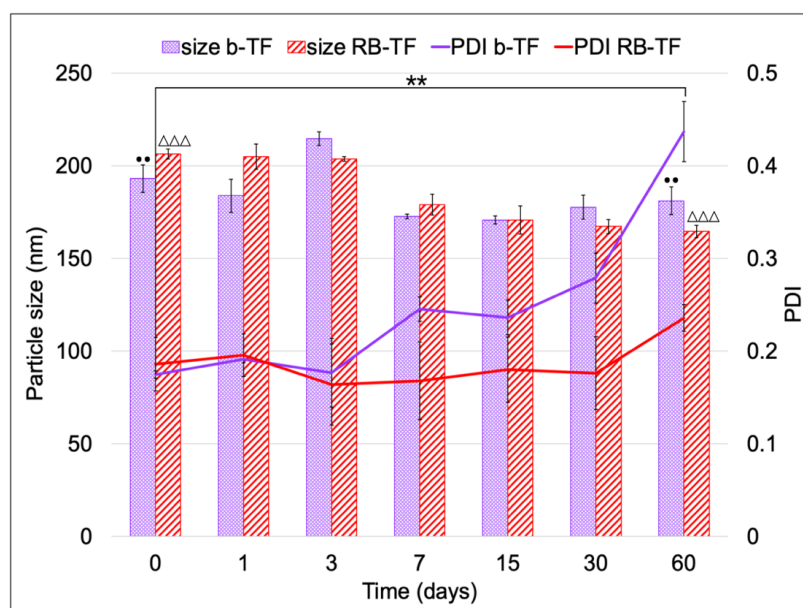


Fig. 7. Storage stability. Particle size and PDI of b-TF and RB-TF following storage at 4°C over 60 days. Results represent the average value of six independent measurements for each formulation (n=6). PDI (**) and particle size (••) of b-TF at 0 days vs. 60 days: p value<0.01; particle size of RB-TF at 0 days vs. 60 days: p value<0.0001(ΔΔΔ).

Encapsulating RB in TF affected the mean particle size and PDI of the system over time. PDI of b-TF increased from 0.175 ± 0.003 nm at the moment of preparation to 0.437 ± 0.032 nm after 60 days ($p < 0.01$) in a progressive manner; PDI of RB-TF proved to be reasonably constant during the first 4 weeks, and an irrelevant increase was observed at 60 days, reaching 0.235 ± 0.015 ($p > 0.05$). Both formulations displayed a reduction in particle size during the storage time: b-TF decreased to 181.08 ± 7.40 nm ($p < 0.01$) and RB-TF to 164.55 ± 3.25 nm ($p < 0.0001$). For RB-TF, parallel to changes in dimensional profile, a reduction of zeta potential was observed: values decreased from -45.2 ± 1.4 mV to -35.6 ± 1.6 mV at 60 days ($p < 0.0001$).

The chemical stability test was performed to determine if any interaction between RB and the components of the formulation could occur over time, leading to a degradation of the drug itself. RB content over time did not vary for both RB aqueous solution and RB-TF dispersion ($p > 0.05$), concluding that no undesired loss of RB occurred, and that RB-TF is chemically stable.

Figure 8 reports the residual amount of RB in RB aqueous solution and RB-TF over 24 hours of irradiation under visible light.

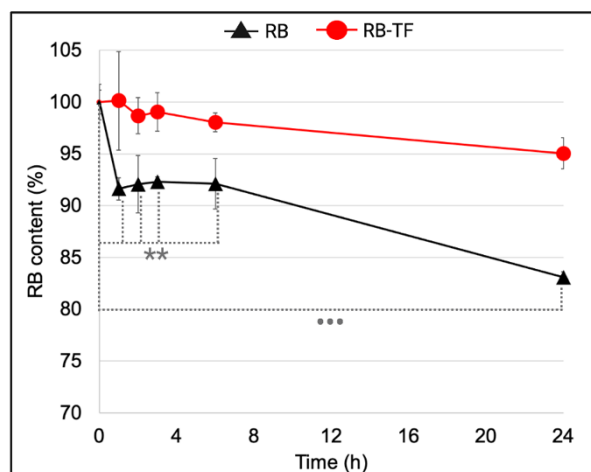


Fig. 8. Photostability study. Residual RB in RB aqueous solution and RB-TF dispersion after 24 hours of irradiation with visible light. The results represent the mean values of three independent experiments for each formulation ($n=3$). Initial free RB amount vs. free RB amount at 1 h, 2 h, 3 h, 6 h: p value <0.01 (**); Initial free RB amount vs. free RB amount at 24 h: p value <0.0001 (***).

Photostability measurements confirmed RB aqueous solution undergoes photodegradation: after 1 hour, RB content decreased to $91.63 \pm 4.75\%$ ($p < 0.01$), at 6 hours, RB content was $92.11 \pm 0.9\%$ ($p < 0.01$) reached $83.09 \pm 1.49\%$ at the end of 24 hours ($p < 0.0001$). On the other side, RB-TF dispersion efficiently protected RB from visible light over 24 hours: at the end of the experiment, RB content in RB-TF was reported to be $95.05 \pm 1.49\%$ ($p > 0.05$).

3.4 *In vitro* release study.

Figure 9 depicts the *in vitro* release profile of RB-TF dispersion and the cumulative amount of RB aqueous solution in the acceptor medium. The results represent the mean values of 3 independent experiments for each formulation over 48 h, following a single dose of RB aqueous solution and RB-TF dispersion (RB=1 mg).

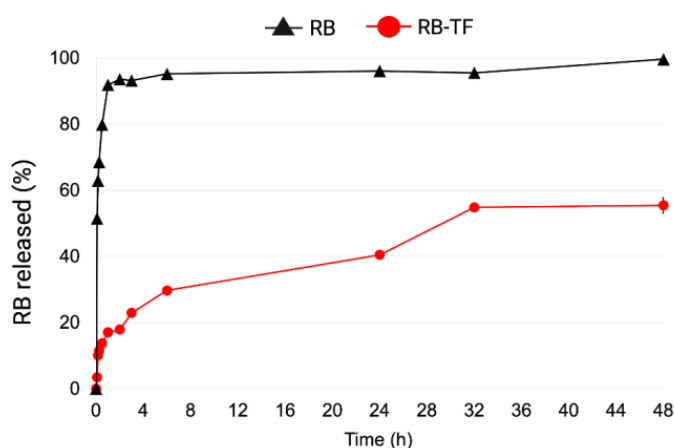


Fig. 9. *In vitro* release profiles of free RB and RB-TF dispersion. The cumulative amount of RB aqueous solution (black line) and RB-TF (red line) recovered in the acceptor medium over 48 h (n=3).

As data proved, when RB aqueous solution was tested, $51.43 \pm 0.15\%$ of the dye was detected within 5 min, and $91.82 \pm 0.3\%$ in 1 h, reaching $99.60 \pm 0.3\%$ at the end of the experiment. On the contrary, the RB-TF dispersion release rate was slower than RB aqueous solution: the maximum RB amount released was $54.81 \pm 0.25\%$ reached after 32 h.

The lack of a burst RB release for RB-TF dispersion, suggests that a significant amount of RB interacted with lipid nanovesicles and thus, it is not in solution such as in RB aqueous solution.

3.5 *Ex vivo* epidermis permeation and retention study.

The *ex vivo* permeation and retention studies were carried out to predict the delivery performance of RB-TF dispersion across the epidermis compared to RB aqueous solution. Figure 10 reports the average values of RB permeated through the epidermis and RB retained over 24 h, following a single dose of RB aqueous solution and RB-TF dispersion (RB=0.5 mg). The results represent the average of 3 independent experiments for each formulation.

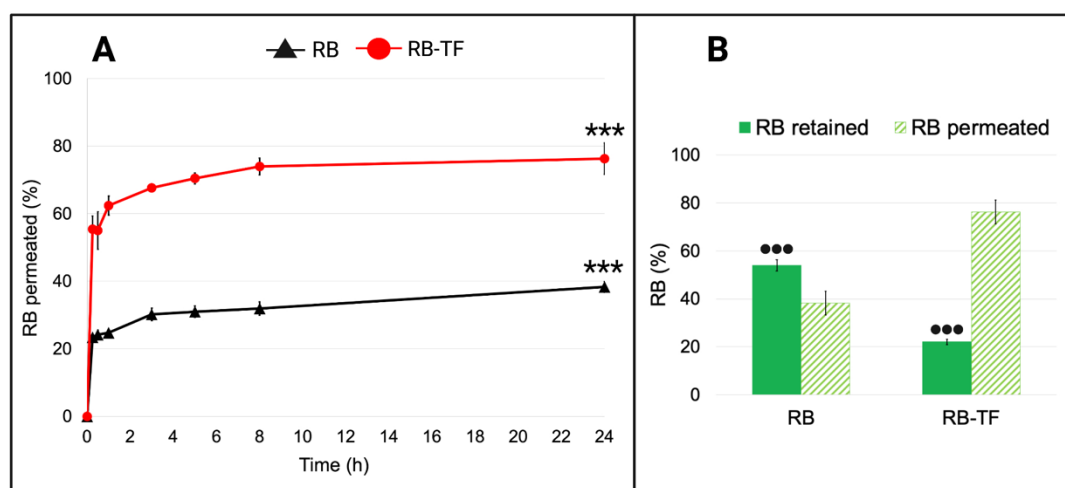


Fig. 10. *Ex vivo* epidermis permeation and retention profiles of RB aqueous solution and RB-TF dispersion. A) Cumulative amount of RB aqueous solution (black line) and RB-TF dispersion (red line) permeated through epidermis over 24 hours (n=3). RB-TF dispersion permeated after 24 hours vs. RB aqueous solution permeated after 24 hours: p value < 0.0001 (***) ; B) Amount of RB aqueous solution and RB-TF dispersion retained by epidermis (full green bar) and totally permeated (striped bar) at the end of *ex vivo* permeation test (n=3). RB-TF retained by epidermis and RB aqueous solution retained by epidermis: $p < 0.0001$ (•••)

The amount of RB aqueous solution permeated through the epidermis was $24.77 \pm 0.88\%$ after 1 h, and it increased to 38.31% at the end of 24 h. On the other side, RB-TF dispersion was found to permeate by $55.40 \pm 3.93\%$ within 15 min, reaching its maximum after 8 h ($73.99 \pm 2.49\%$). At 24 h, an irrelevant increase of RB-TF dispersion permeation was observed ($p > 0.05$). In parallel, the drug

amount detected in the epidermal layer after 24 h was significantly different for RB aqueous solution and RB-TF dispersion ($p<0.0001$): $54.00\pm2.37\%$ of RB aqueous solution was retained by the skin, whereas RB-TF dispersion allowed decreasing RB retention to $22.08\pm1.14\%$. *Ex vivo* permeation study proved that RB-TF dispersion doubled RB's amount that permeates the epidermis in 24 h, limiting the skin deposition phenomena.

3.6 Cytotoxicity and antiproliferative studies.

The impact of RB aqueous solution and RB-TF on cell death and viability was evaluated on SK-MEL28 and HFFF2 cells (Figure 11). After 48 h, no cell death induced by the formulations was observed (data not shown). Both cell lines displayed a dose-dependent reduction of growth response to RB aqueous solution and RB-TF. Data suggested that both at a concentration of 25 and 50 μM , RB-TF cell growth reduction was significantly higher than RB aqueous solution ($p<0.05$). At the concentration of 50 μM , RB aqueous solution inhibited the percentage of growth of SK-MEL 28 and HFFF2 without a selectivity ($p>0.05$); on the other hand, RB-TF displayed a potential selectivity for SK-MEL28 at this concentration ($p=0.05$). On the contrary, no growth reduction was noticed in b-TF, which instead promoted cell growth.

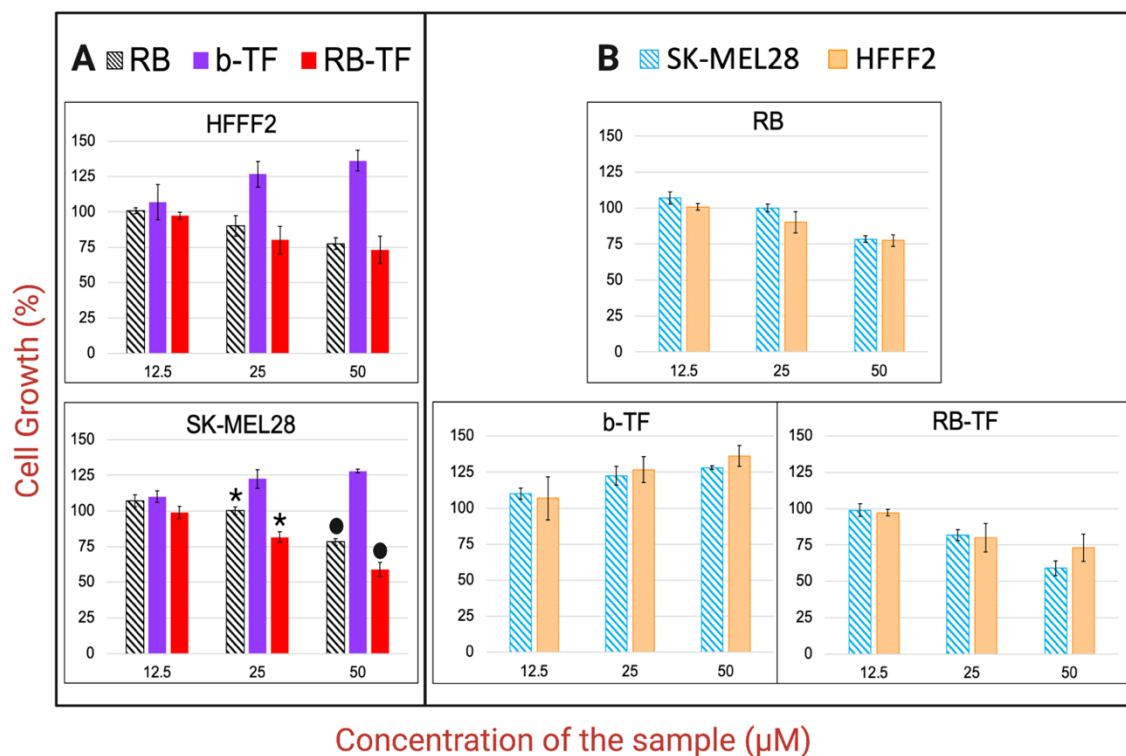


Fig. 11. Effect of RB aqueous solution, RB-TF, and b-TF on SK-MEL28 and HFFF2 cell growth. Cells were cultured with samples for 48 hours. Figure A displays viability of each cell line depending on the formulation

and the concentration tested. Figure B analyzes each formulation's impact at different concentrations, comparing the growth suppression of the two cell lines. Effect of RB aqueous solution (25 μ M) on SK-MEL28 vs. the effect of RB-TF (25 μ M) on SK-MEL28: p value<0.05 (*). Effect of RB aqueous solution (50 μ M) on SK-MEL28 vs. the effect of RB-TF (50 μ M) on SK-MEL28: p value<0.05 (•).

4. Discussion.

The current work aimed to determine if a proper delivery system can enhance the anti-melanoma intrinsic activity of Rose Bengal. Considering the localization of primary melanoma tumour, the dermal administration was here investigated as it represents the most direct and non-invasive access route to cancer cells, obviating the need for intralesional injection. We developed deformable lipid nanovesicles to vehicle RB across the epidermis and to reach the dermal layer. In this way, RB-TF could potentially eradicate melanoma lesions at early stages localized in the outmost layer of the skin (melanoma in situ) and localized but invasive melanoma, which advances deeper in the dermis [32].

Based on RB disodium salt high water solubility, TF were prepared using modified reverse phase evaporation (REV) technique [20]. This method allows obtaining vesicles with a high aqueous space-to-lipid ratio and, consequently, to entrap a large percentage of drug solubilized in the aqueous phase [33]. The original REV technique, first reported in 1978 by Szoka et al. [34], studied several phospholipids, either pure or mixed with other lipids, mainly involving diethyl ether, isopropyl ether, and chloroform as solvents. Herein, the lipid phase was made of phosphatidylcholine, cholesterol, and Span® 80 as the surfactant. Cholesterol was added to improve liposomes' resistance to leakage and degradation *in vivo*, as is known to provide rigidity to the structure [35]; simultaneously, the presence of the surfactant destabilizes the lipid bilayer, increasing the vesicle's deformability. As previously reported by Joshi et al. [36], a proper balance of cholesterol and surfactant can provide the desired flexibility to the lipid vesicle to improve the migration towards the deepest region of the skin. Moreover, the surfactant can improve properties such as encapsulation efficiency, stability, and permeability [15,37]. Besides, ethanol has been employed to replace solvents originally used (supporting information) to obtain a formulation as safest as possible. Ethanol is a solvent with low toxic potential (Pharmacopoeia Italica, XII ed.), whereas the toxicity of ethers and halogenated hydrocarbons is well known [38]. To optimize the dimensional properties of nanoparticles, formulations were finally sonicated and extruded (supporting information). Probe sonication reduced particle size, as previously reported by Hadian et al. [39], who evaluated the effect of sonication on similar lipid vesicles. Since lipid components here used are heat-sensitive, the

formulations were sonicated in cycles to permit TF to cool and avoid thermal degradation [21]. The extrusion process allowed a narrow particle size distribution because it forced the dispersion to pass several times through a membrane with uniform pores [25].

The final TF dispersions appeared turbid and milky, indicating that average particle size was above 100 nm; indeed, formulations having an average particle size of 100 nm or smaller are instead characterized by a transparent, bluish appearance (Tyndall effect) [40]. PCS later confirmed dimensional properties: b-TF and RB-TF had a particle size around 200 nm. Such a result agrees with a study conducted by Godbole et al. [41], reporting that a milky appearance characterizes liposomes above 300 nm and those ranging between 200-300 nm. Also, even if the average size of formulations was around 200 nm and PDI below 0.2, such values do not exclude the presence of vesicles larger than 200 nm, affecting the milky appearance of the dispersion. Lipid vesicles whose size was between 100–600 nm proved to enhance skin penetration of water-soluble photosensitizer drugs, and in particular, TF ≤ 300 nm reach deeper into the skin [13,15].

Zeta potential is a fundamental parameter to predict the system's stability, as it measures the magnitude of the repulsion between charged particles in the dispersion. The higher zeta potential value denotes highly charged particles and minimizes the possibility of aggregation via electrostatic interaction. Conventionally, a sample exhibiting a zeta potential value higher than $|30|$ mV is considered stable [25]. Zeta potential measurement showed that TF's surface charge significantly decreased following RB loading, reaching -45.9 mV. As previously observed for Indocyanine Green zein-phosphatidylcholine nanoparticles, this suggests that the negatively charged RB was incorporated in the phospholipid bilayers, and the incorporation improved the electrostatic stabilization of the system [42]. Such a result agrees with the storage stability observed for b-TF and RB-TF. The PDI value of b-TF increased from the preparation. In contrast, the PDI of RB-TF remained relatively constant, implying that b-TF progressively aggregates over time and that RB presence limits the aggregation phenomena.

The stability of RB-TF was also studied in terms of drug content over time and photostability. Over time, no reduction in RB content was noticed, evidencing the chemical compatibility between the formulation and the drug components. On the contrary, we observed that photodegradation of RB aqueous solution was significantly faster than RB-TF dispersion and that the last one efficiently protected RB over 24 h. As already observed by Ali et al.[43], this difference can be related to the light scattering exerted by lipid, reducing the photon energy delivered to RB associated with

vesicles. Because the dermal administration is an administration route exposed to visible light, protecting RB is essential to avoid an undesirable drug loss.

Morphological investigation (TEM) revealed MLV b-TF and LUV RB-TF, as also proved by SAXS analysis. The formation of LUV is a consequence of the RB presence, which is likely to intercalate in the hydrophobic palisade of the phospholipid bilayer, thus modifying the effective lipid packing parameter $P = v/a_0l_c$, where v represents the volume of the hydrophobic chain, a_0 is the headgroup area, and l_c is the critical hydrocarbon chain length (approximately equal to the fully extended chain length) [44,45]. Such alteration of P can be undoubtedly called into play to explain the different lamellarity degree observed in b-TF and RB-TF and may also play a role in the different behavior of nanovesicles in terms of deformability. This section previously discussed that cholesterol provides rigidity to the liposomal structure; only a proper balance of cholesterol and surfactant can provide the desired flexibility leading to a deformable formulation. Dudhipala et al. [46] observed that the optimal concentration of Span®80 to obtain the best deformability index for aceclofenac-transferosomes is 0.15% w/v and that higher concentrations reduce the deformability. The authors hypothesized that this is due to the linear structure of the Span®80, which tends to compact the double layer and, therefore, reduce the TF's deformability. This evidence and the presence of cholesterol can explain the non-deformability of b-TF. On the other side, Bouvrais et al. [47] proved that the bending rigidity of lipid membranes strongly depends on the organization of lipid bilayers, which can be modified by additives or environmental modification, e.g., by introducing salt and buffers. In agreement with deformability indexes observed, it can be assumed that adding RB to the lipid bilayer induced a softening effect on b-TF, resulting in unilamellar and deformable RB-TF.

The spectrophotometric and fluorimetric analyses further confirmed the interaction between RB and lipid components. For RB-TF dispersion and RB added to b-TF dispersion, the RB absorption λ_{max} shifted to a higher wavelength than RB aqueous solution. Since the dye's solvatochromic behavior has been reported, λ_{max} is indicative of the microenvironment interacting with RB when most of the dye is bound to the dispersed phase [48]. The redshift observed in the absorption spectra implies that RB sensed the less polar environment of TF and migrated. Also, the emission spectra of RB-TF dispersion showed an increase in the fluorescence signal. As previously reported by Chang et al. [27], the higher intensity ratio suggests that free RB intercalated through the lipid bilayer mainly as a monomeric form but not as a dimer form, effectively limiting RB aggregates' formation. Nevertheless, the logarithm of the partition coefficient ($\log P$) of RB disodium salt is reported to be 0.59, resulting in an amphiphilic molecule [6]. Considering the amphiphilic properties

and high water solubility, the distribution of RB both in the external lipid bilayer and in the internal aqueous core of the TF is expected.

The above characterizations agree with *in vitro* release and *ex vivo* permeation profiles obtained experimentally. *In vitro* release assay proved that RB aqueous solution quickly diffuses through the hydrophilic 50 nm size polycarbonate membranes, whereas RB-TF dispersion released RB slower and in a controlled manner. In contrast, the ability of RB aqueous solution to permeate the epidermis and consequently reach the dermis was limited by RB's physical-chemical profile. The epidermis is the outmost layer of the skin; it comprises different living epithelial cell strata (viable epidermis) localized beneath a multilayer of dead cells called corneocytes (stratum corneum, SC). As previously stated in the introduction, SC is considered the main barrier against the penetration of external agents [11], significantly when molecules exceed 500 Dalton and present anionic or cationic charges [12,15]. RB is an amphiphilic drug, but it presents two anionic charges and a molecular weight of 1017.64 g/mol [6]. As the *ex vivo* skin permeation study proved, less than 40% of RB aqueous solution permeated through the epidermis, and most of it was retained by the epidermal layer. Formulating RB in TF doubled RB's amount that permeated the epidermis and effectively reached the dermal layer. Herein, no studies evaluating the mechanism of RB-TF's permeation were performed. However, due to their deformability, the intact permeation of nanovesicles through the skin is supposed to be the primary operating action. In support of this hypothesis, the elastic properties of RB-TF were assessed and previously discussed. Nevertheless, other mechanisms of action cannot be excluded; these include the structural modification of the intracellular lipid matrix due to the penetration enhancing effect of the surfactant and the vesicles adsorption to and/or fusion with the stratum corneum [18,19].

The cytotoxicity of RB and RB-TF in SK-MEL28 cells was determined after 48 hours of exposure. Although RB is mainly considered for its photodynamic inactivation of cancer and microbial cells, previous evaluations of its intrinsic cytotoxicity were reported. It was observed that RB concentrations ranging between 5-10 μM induce cell death only following RB photosensitization. RB intrinsic toxicity on melanoma and breast cancer cell lines was observed at a concentration of 100 μM ; in particular, it was evident for melanoma in the case of 200 μM and for breast cancer at 300 μM [8,49]. Such results agree with the values obtained in SK-MEL28 cells. Herein, we evaluated RB-induced toxicity up to a maximum of 50 μM in the absence of external stimuli, including light or ultrasounds. At this concentration, a significant cytostatic effect was observed. Based on this outcome, we also investigated the efficacy of RB-TF to reduce the viability of SK-MEL28. The

cytostatic effect of RB-TF was higher than free RB, and the explanation is based on the chemical profile of RB. RB is an amphiphilic water-soluble compound with a more hydrophilic tendency due to two negative charges at physiological pH; these characteristics make RB hard to cross lipophilic cell membranes spontaneously, resulting in an insufficient cell accumulation at low concentrations in the absence of a carrier. We hypothesized that the association of RB with the lipophilic TF increased RB cell uptake and consequently the cytostatic effect[6]. On the other side, b-TF did not induce any effect supporting the thesis of a safe nanocarrier enhancing RB anti-melanoma activity. Besides, at 50 μ M, a promising selectivity of RB-TF on cancer cells compared to fibroblast was noticed.

5. Conclusion.

We have developed RB-loaded transfersomes to combine the permeation ability of deformable lipid vesicles with the dye's intrinsic anti-melanoma activity. The preparation technique employed (REV) avoided using toxic organic solvents and allowed to obtain monodispersed nanoparticles in a dimensional range suitable for dermal delivery; RB efficiently associated with the lipid carrier. *Ex vivo* epidermis permeation confirmed that TF significantly improve the permeation of RB across the epidermal barrier. Furthermore, *in vitro* antiproliferative assays indicated a higher intrinsic cytostatic effect of RB-TF compared to free RB. Considering these outcomes, RB-TF represents a promising approach to fight against primary cutaneous melanoma without involving sonophotodynamic treatments. Future studies will involve the development of an RB-TF controlled release dosage form and the evaluation of dermatokinetic vs. its pharmacokinetic when administered intravenously.

CRediT authorship contribution statement

Sara Demartis: *Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Visualization.* **Giovanna Rassu:** *Methodology, Writing – review & editing.* **Sergio Murgia:** *Methodology, Investigation, Writing – review & editing.* **Luca Casula:** *Investigation.* **Paolo Giunchedi:** *Writing – review & editing.* **Elisabetta Gavini:** *Conceptualization, Resources, Writing – review & editing, Supervision.*

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7. Supporting material

Preformulation study of deformable lipid vesicles.

Materials. Rose Bengal sodium salt (RB), cholesterol, Span® 80, chloroform, ethanol, methanol and dyethyl ether were purchased by Sigma-Aldrich (St. Louis, MO). Lipoid S 100 was gifted by Lipoid GmbH (Ludwigshafen, Germany). Ultrapure bi-distilled water was obtained by a MilliQ R4 system, Millipore (Milan, Italy).

Methods. Transfersomes (TF) Preparation. Two series of TF were developed, TF1 and TF2. About TF1, TF1-A and TF1-B were studied (Table 1). TF1-A were prepared by thin-film hydration method (TFH), TF1-B by reverse-phase evaporation method (REV). 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. The leader formulation (RB-TF) was prepared based on TF1 study changing solvent and sonication time (Table 2). RB-TF 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 (RB=500 µM) sonicated for 30 s 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 20 s. RB-TF were extruded 10 times through a regenerated cellulose syringe filter (pore size: 0.45 µm, filter size: 25 mm, AlfaTech, Genova, Italy). Corresponding blank (b-TF) was prepared.

Table 1. *Preformulative parameters modified during TF preparation.*

<i>Formulation</i>	<i>Technique</i>	<i>Solvents</i>	<i>Sonication</i>
TF1-A	TFH	Ethanol Methanol Chloroform	Probe 50% (60 s, 90 s, 120 s) Bath 40°C (5', 10', 15')
TF1-B	REV	Diethylether Chloroform	Probe 50% (60 s, 90 s, 120 s) Bath 40°C (5', 10', 15')
TF-2	REV	Ethanol	45% US

Particle size analysis. Dimensional analysis of formulations was performed by photon correlation spectroscopy (PCS). A Coulter nanosizer N5 (Beckman-Coulter Inc., Miami, FL, USA) was used to determine the hydrodynamic diameter and the polydispersity index (PDI) to measure the dimensional heterogeneity of the sample. Before each determination, all samples were diluted adequately with MilliQ water previously filtered (regenerated cellulose syringe filter, pore size: 0.20 µm, filter size: 15mm, Albet LabScience, Dassel, Germany) to ensure light-scattering intensity within the required range of the instrument (between 5×10^4 and 1×10^6 counts s⁻¹). Particle size was calculated in unimodal using the following conditions: fluid refractive index 1.333; temperature 25°C; viscosity 0.890 centipoises; angle of measurement 90°; sample time 3.0 ms and sample run time 300 s.

Results. Figure 1 shows the effects of sonication on TF1 dimensional profile. 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 1µm, 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, RB-TF were prepared by REV technique and probe sonication method.

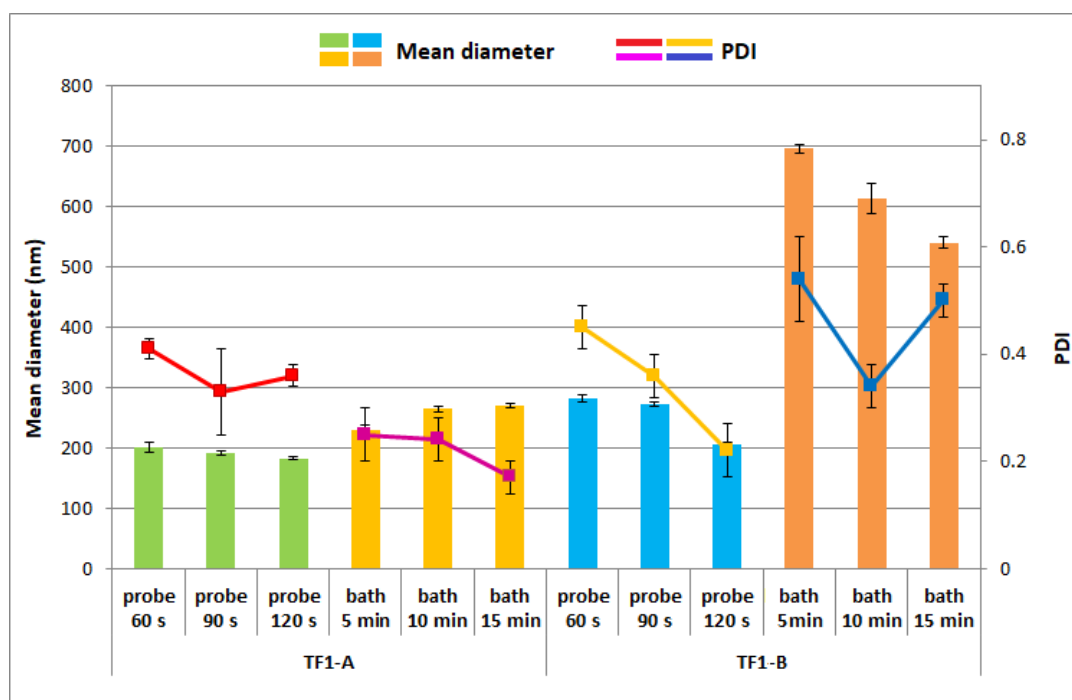


Fig. 1S. Influence of sonication technique and time on TF1 dimensional profile.

***In vitro* release study.**

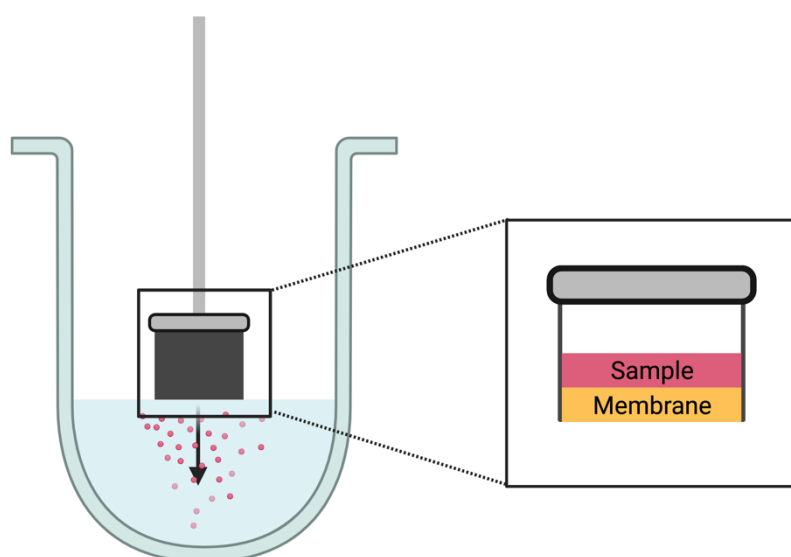


Fig. 2S. Schematic illustration of the dissolution apparatus employed to perform the *in vitro* release study. *In vitro* release profile of RB-TF dispersion was evaluated across a polycarbonate membrane (0.050 μm pore size, 47 mm diameter, Sigma-Aldrich, St. Louis, MO, USA) using a dissolution apparatus (DT 70, Erweka, Langen, Germany), as previously described by Gavini et al.²⁸. The membrane was mounted on the bottom of cylindrical plastic support consisting of a tube (height=1.91 cm, diameter=2.28 cm) connected to a drive shaft of the dissolution apparatus. The membrane was clamped to the support by a plastic ring, and then 2 mL of the sample (1 mg of RB) were placed on the surface of the membrane. The system was then inserted into the vessel containing 250 mL of PBS as the acceptor medium, keeping the membrane in contact with the surface of the PBS all the time. RB aqueous solution was also tested as comparison.

Research Article

Fluorescence-coupled techniques for the determination of Rose Bengal in dermatological formulations and their application to ex vivo skin deposition studies.

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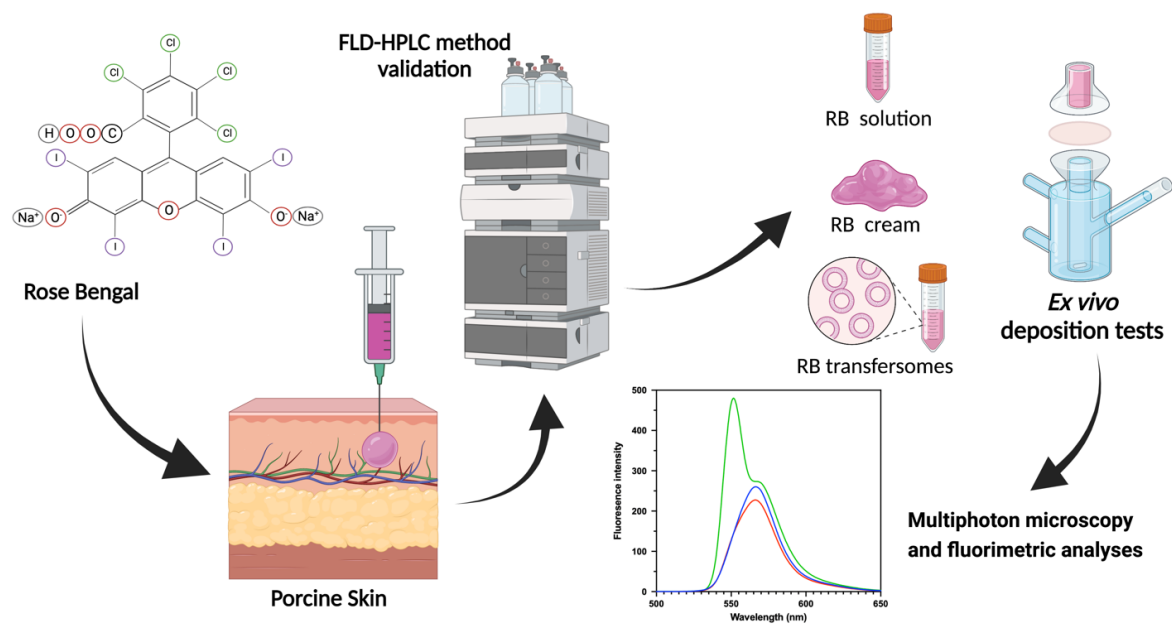
<https://doi.org/10.3390/pharmaceutics15020408>

The current experimental section describes the quantitative and qualitative methodologies that have been used now on in the presented Thesis to determine Rose Bengal in the skin tissue. The methodologies herein presented have been developed during the second and third Academic Years of the PhD, during the visiting to the Prof. Ryan Donnelly's lab (School of Pharmacy Queen's University Belfast). Lastly, the investigation was published as an Original Research Article titled *"Fluorescence-coupled techniques for the determination of Rose Bengal in dermatological formulations and their application to ex vivo skin deposition studies"* in the peer-reviewed and indexed journal *"Pharmaceutics"*; (2021 Journal Impact Factor (JIF): 6.525; JIF Quartile: Q1 in the category Pharmacology and Pharmacy).

Abstract

Rose Bengal (RB) is a fluorescent dye with several potential biomedical applications, particularly in dermatology. Due to RB's poor physicochemical properties, several advanced delivery systems have been developed as a potential tool to promote its permeation across the skin. Nevertheless, no validated quantitative method to analyse RB within the skin is described in the literature. Considering RB exhibits a conjugated ring system, the current investigation proposes fluorescence-based techniques beneficial for qualitatively and quantitatively determining RB delivered to the skin. Notably, the development and validation of a fluorescence-coupled HPLC method to quantify RB within the skin matrix are herein described for the first time. The method was validated based on the ICH, FDA and EMA guidelines, and the validated parameters included specificity, linearity, LOD, LLOQ, accuracy and precision, carry over and dilution integrity. Finally, the method was applied to evaluate RB's *ex vivo* permeation and deposition profiles when loaded into dermatological formulations. Concerning qualitative determination, multiphoton microscopy was used to track the RB distribution within the skin strata, and fluorescence emission spectra were investigated to evaluate RB's behaviour when interacting with different environments. The analytical method proved specific, precise, accurate and sensitive to analyse RB in the skin. In addition, qualitative side-analytical techniques were revealed to play an essential role in evaluating the performance of RB's dermatological formulation.

Keywords: Rose Bengal; skin; fluorescence detection; method validation; *ex vivo* permeation tests; topical dosage forms; multiphoton microscopy.



1. Introduction

Rose Bengal (RB) is a violet dye synthesised from fluorescein in the 19th century as a wool colourant. Chemically, RB, the 3', 4', 5', 6' -tetrachloro-2,4,5,7-tetraiodo-fluorescein is a highly water-soluble (100 g/L), fluorescent dianionic molecule belonging to the class of xanthene dyes [1]. Beyond its colouring abilities, RB exhibits intrinsic cytotoxicity towards different cancers [2–6] and microbial cells [7] and sono-photochemical properties, which allow its application in photodynamic (PDT) [8–12] and sonodynamic therapy (SDT) [13–15].

In recent decades, RB has garnered the scientific community's interest, particularly in its potential role in managing dermatological diseases. Due to its intrinsic cytotoxicity, RB has been widely investigated as a potential chemotherapeutic in treating cutaneous melanoma, both early and metastatic [2,16,17]. To date, RB is undergoing clinical trials for the treatment of melanoma with the name of PV-10® (Provectus Biopharmaceuticals, INC), which is a 10% w/v RB sterile and nonpyrogenic saline solution suitable for intralesional (IL) administration [18]. Despite its promising results, IL administration requires the healthcare assistance of professionals and causes significant pain and discomfort to the patient, which may result in poor compliance. More importantly, systemic phototoxicity following the intralesional administration of RB has been reported as a side effect [19]. Nevertheless, the IL route appears to be the most efficient administration considering RB's high water-solubility and low permeability [1,15].

PH-10® is a topical RB hydrogel formulation that selectively delivers RB to epithelial tissue and is currently involved in clinical trials [20]. PH-10® has been employed for managing psoriasis and atopic dermatitis with encouraging results. Nevertheless, the action mechanism of RB in this disease has yet to be elucidated. Furthermore, RB, alone or in combination with stimuli-responsive therapies, has been reported to be a promising candidate for a plethora of therapeutic applications ranging from photochemical tissue bonding [20–22], white hair removal [23], plantar warts [24], and eradication of certain skin infections [7,25–27].

To obviate RB shortcomings, including low bioavailability and scarce permeation of biological membranes, research has been focused on developing advanced drug delivery systems that could help augment the efficacy of RB in dermatology (figure 1) [1,28]. Some of these drug delivery systems include liposomes [29], transfersomes [30], micellar platform [31], microemulsion [32],

hydrogel [33], upconversion particles [34,35], inorganic nanoparticles (NP) [36,37], hybrid NP [24], dissolving polymeric microneedles [38].

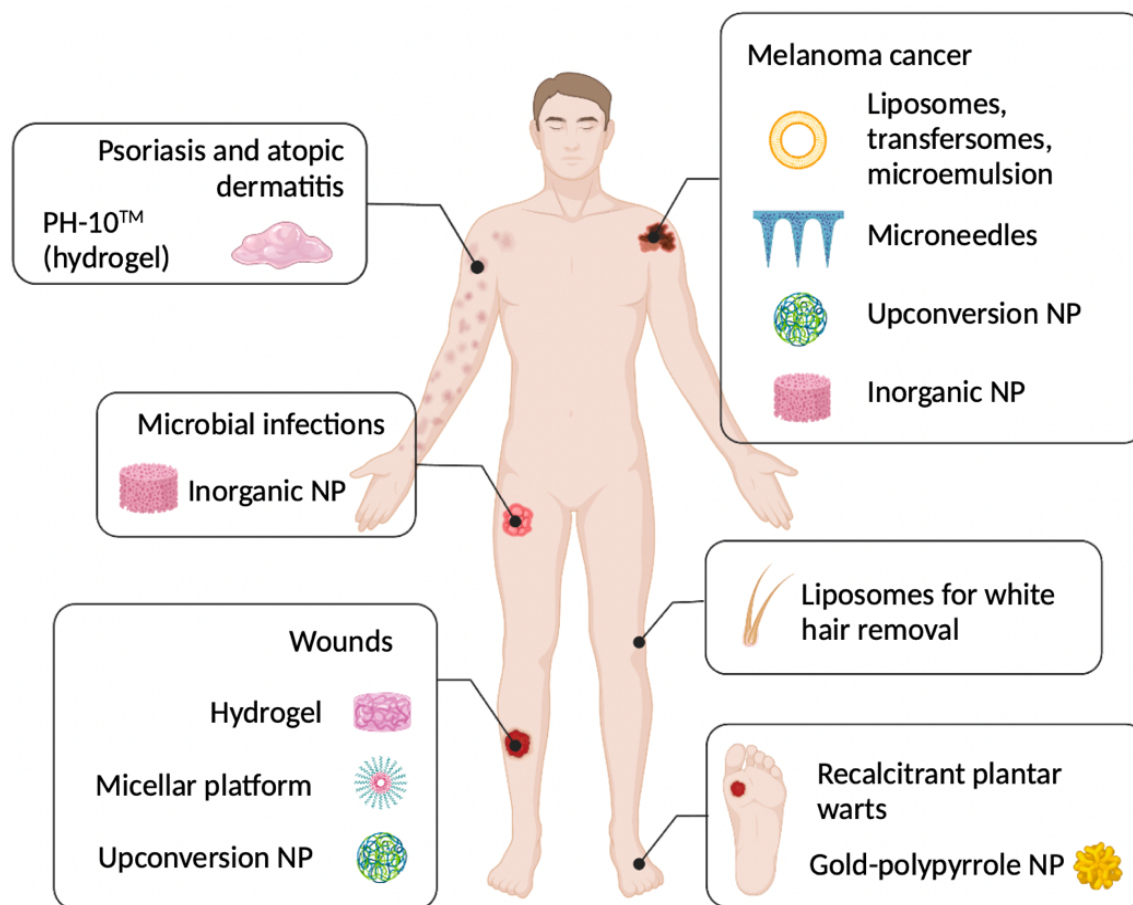


Fig. 1. Summary of applications of RB-loaded formulations in managing dermatological diseases from the literature.

Despite the role of RB in dermatology and the continuous investigation of advanced drug delivery systems, no validated quantitative method to analyse RB within the skin has been described in the literature. The detailed analysis of a drug within the skin strata is essential in estimating and understanding the permeation enhancement effect conferred by the delivery system used. RB is easily quantifiable using UV-visible and fluorescence techniques due to the presence of a conjugated ring system in its structure. The maximum wavelengths at which RB absorbs and emits have been determined in different solvents and are reported to be 546 nm and 567 nm in water, respectively [39]. To the best of our knowledge, only one validated RB quantitative method has been described, exploiting RP-HPLC with UV detection at 262 nm to quantify the analyte within eye surgical strips and not within the skin layers [40]. Other investigations mainly employed the UV-Vis spectrophotometer technique to determine RB content [31,41–44]. Many dermatological

formulations contain biopolymers and lipids that must be removed before the analysis to mitigate any potential interference or matrix effect, which may interfere with RB quantification [45]. However, based on the chemical profile of RB, the complete isolation from excipients is not always successful, and some undesired traces can remain in the sample for analysis. RB strongly interacts with phospholipids, and its absorption and emission spectra are influenced by this interaction [30,46], as well as by the interaction with polymers [47] or skin components themselves [31]. In those cases, UV-vis techniques may suffer from poor selectivity, sensitivity, and relatively high inconstancy [39,48].

Fluorescence-coupled techniques have been widely used to evaluate the efficiency of drug delivery systems intended to be administered to the skin [49]; wide-field fluorescence and multiphoton microscopies are some of those techniques. Pena-Rodríguez and collaborators exploited the wide-field fluorescence microscopy to determine the biodistribution of Retinyl Palmitate Loaded Transfersomes within the skin layers [49], aiming the epidermal delivery; similarly, Mangion et al. [50] employed multiphoton microscopy to assess the follicular delivery of zinc pyrithione. Due to its intrinsic fluorescence, RB can be easily tracked within a biological milieu by means of the techniques mentioned above. In this regard, it has been reported that staining hepatoma cells with RB increased their visualisation under multiphoton microscopy compared to other dyes [51]. Besides that, fluorescence-based microscopies have been a helpful armamentarium for evaluating the dermatokinetic of topically applied RB [52]. Recently, we have exploited the multiphoton technique to estimate the penetration depth of an RB microneedle formulation into the full-thickness porcine skin [38].

Based on this rationale, this study intends to investigate the fluorescence-coupled techniques that should be considered to analyse RB in the skin, thus supporting the successful development of a skin-targeted delivery system. Two aims have been pursued in this work: (i) validating a selective and sensitive HPLC method for the RB quantitative determination in the skin matrix; (ii) offering side-analyses techniques to evaluate the performance of an RB formulation qualitatively. Herein, we report, for the first time, the validation of an analytical method for RB quantification using the HPLC technique coupled with a fluorescence (FLD) detector. The validated analytical method was then applied to an *ex vivo* skin deposition experiment comparing three RB topical formulations (aqueous solution, cream and transfersome dispersion); the HPLC results were then cross compared with multiphoton microscopy results. For this, the fluorescence spectra of skin spiked with RB and RB loaded in different formulations were acquired.

2. Materials and Methods

2.1 Materials

Rose Bengal disodium salt (RB), cholesterol, Span® 80, ethanol, HPLC grade methanol, ammonium acetate, sodium hydroxide, phosphate-buffered saline (PBS) tablets and dimethyl sulfoxide (DMSO) were purchased by Sigma-Aldrich (St. Louis, MO). Aqueous cream BP was purchased under the brand name XBC aqueous cream (Buckinghamshire, UK). Ultrapure water was obtained from a water purification system (Elga PURELAB DV 25, Veolia Water Systems, Dublin, Ireland).

2.2 Instrumentation and chromatographic conditions

The apparatus employed to develop the proposed method was an Agilent Technologies 1220 Infinity compacted LC Series equipped with fluorescence (FLD) and UV-vis detector with a binary pump, degasser and standard auto-injector set at room temperature (Agilent Technologies UK Ltd, Stockport, UK). The analyses of RB were performed on a C18 Phenomenex SphereClone® column ODS(1) (150 x 4.6 m) with a particle size of 5 µm and pore size of 100 Å (Phenomenex Cheshire, UK). All chromatograms were recorded and collected using the Agilent ChemStation® Software B.02.01. For this study, we wanted to compare two standard analytical detectors to evaluate which detector conferred the most significant sensitivity. Upon identifying the most sensitive detector, this detector will be used to develop further and evaluate the analytical method for quantifying the molecule of interest, RB. The mobile phase was a mixture of methanol and 20 mM ammonium acetate buffer solution at pH 8, used after degasification and filtration through membrane filters of mixed cellulose esters, 0.45 µm pore size and 47 mm diameter (GE Healthcare Life Sciences, Buckinghamshire, UK). The pH 8 buffer was obtained by accurately weighing and solubilising 1.54 g of ammonium acetate in ultrapure water in a 1000 mL volumetric flask. The pH was adjusted to 8 using NaOH. The isocratic elution of the mobile phase kept 60% methanol and 40% buffer pH 8.0, with a flow rate of 1 mL/min. The excitation wavelength was set at 549 nm concerning UV-Vis detection. Regarding FLD detection, the excitation and emission wavelengths were set at 556 nm and 573 nm, respectively. The injection volume was 40 µL, and the run time was 7 min for UV-vis detection and 6 min for FLD detection. The retention time was 3.1 min for UV-vis and 4.1 min for FLD.

2.3 Standard stock, working solution and calibration standards.

To prepare the standard stock solution, 20 mg of RB were accurately weighed and dissolved in phosphate-buffered saline at pH 7.4 (PBS) into a 20 mL volumetric flask, reaching a 1 mg/mL concentration. One mL of the stock standard solution was withdrawn and further diluted up to 10 mL with PBS to obtain the standard working solution of 100 µg/mL final concentration. From the standard working solution, serial dilutions with PBS were performed to get seven calibration standards ranging from 1.33 to 80 µg/mL in the case of UV-vis determination and from 0.16 to 10 µg/mL in the case of FLD determination.

2.4 Validation process

To ensure that the developed method fit the intended purpose, it was validated based on International Council on Harmonization (ICH) guideline, US Food and Drug Administration (FDA) guideline, and the European Medicine Agency guideline [53–55].

2.4.1 HPLC UV-vis method validation

Concerning the HPLC method coupled with the UV-Vis detector, validated parameters were linearity, limits of detection (LOD) and quantitation (LOQ). Linearity was determined by evaluating the calibration curves from the calibration standards mentioned in section 2.3. Six replicates of the calibration standards were analysed over three consecutive days (n = 6). The instrument response (peak area) was fitted as a function of the theoretical concentration, and the linear regression method was employed to determine the slope, y-intercept, and coefficient of determination (R^2) using GraphPad Prism® 9.4.1 (458). The LOD and LOQ were calculated from equations 1 and 2, respectively, based on the standard deviation of the response and the slope from six calibration curves:

$$LOD = \frac{3.3\sigma}{S} \quad (1)$$

$$LOQ = \frac{10\sigma}{S} \quad (2)$$

Where σ is the standard deviation of the response and S is the slope of the calibration curve.

2.4.2 HPLC FLD method validation

Concerning HPLC-FLD determination, validated parameters were specificity, linearity, LOD and LOQ, inter-day and intra-day accuracy and precision.

2.4.3 Specificity

The specificity was investigated by comparing the chromatograms of six samples of blank PBS, blank samples from skin extract, PBS and skin samples spiked with a known amount of RB. The skin was prepared as reported below in section 2.5.

2.4.4 Linearity, LOD and LLOQ

Linearity, LOD and LLOQ were calculated, as described above in section 2.4.1., using the calibration standards mentioned in section 2.3.

2.4.5 Inter-day and intra-day accuracy and precision

Quality control (QC) solutions were prepared and analysed to calculate inter-day and intra-day accuracy and precision. Following the same procedure described in section 2.3, three quality control (QC) solutions were prepared (1 µg/mL for low QC, 4 µg/mL for medium QC and 8 µg/mL for high QC). The inter-day accuracy and precision were determined by analysing the QC solutions within one run (n = 6); Intra-day accuracy and precision were observed between runs over three consecutive days (n= 6). The precision was expressed as the relative standard deviation (RSD%) of the responses of all samples (equation 3); the accuracy test indicated the relative error (RE%) between the experimental measurements and the theoretical concentrations (equation 4). The RSD% and RE% maximum values were set at 15%.

$$RSD\% = \frac{\text{Standard deviation of experimental measurements}}{\text{Mean of experimental measurements}} \times 100 \quad (3)$$

$$RE\% = \frac{\text{Absolute error}}{\text{Theoretical concentrations}} \times 100 \quad (4)$$

Where the absolute error represents the deviation between experimental measurements and theoretical concentrations.

2.4.6 Carry-over

In order to evaluate the carry-over of RB, QC sample at high concentration was injected, followed by the blank solution. The response of the blank solution obtained should not be more than 20% of the response from the sample at LLOQ concentration [54,55].

2.4.7 Dilution integrity

Dilution integrity was assessed by injecting the diluted samples (5 and 10 times) in PBS (pH 7.4) media. The accuracy and precision of obtained responses were then calculated [54,55].

2.4.8 Skin extraction recovery

RB's extraction recovery from dermatomed neonatal porcine skin was determined by analysing skin samples spiked with QC solution. The skin was obtained from stillborn piglets from Agri-Food and Bioscience Institute (Hillsborough, Northern Ireland, UK); the newborn piglets were instantly frozen at -20°C defrosted overnight before experimentation. First, full-thickness skin was excised using a surgical scalpel and cautiously shaved using a disposable razor (Gillette Blue II™, Gillette, Reading, UK). Then, dermatomed skin was isolated by trimming the shaved skin using an electric dermatome (Integra Padgett® model B, Integra LifeSciences Corporation, Ratingen, Germany) to a thickness of around 350 μm . The skin was finally allowed to equilibrate for 30 minutes in PBS before the study. To perform the skin extraction recovery test, as illustrated in figure 2, RB solutions were incubated with 100 mg of porcine skin in a 2 mL Eppendorf tube at $37 \pm 0.5^{\circ}\text{C}$ for 24 hours (Drying Oven VWR™ VENTI-Line™ VL 115, VWR International BVBA, Leuven, Belgium).

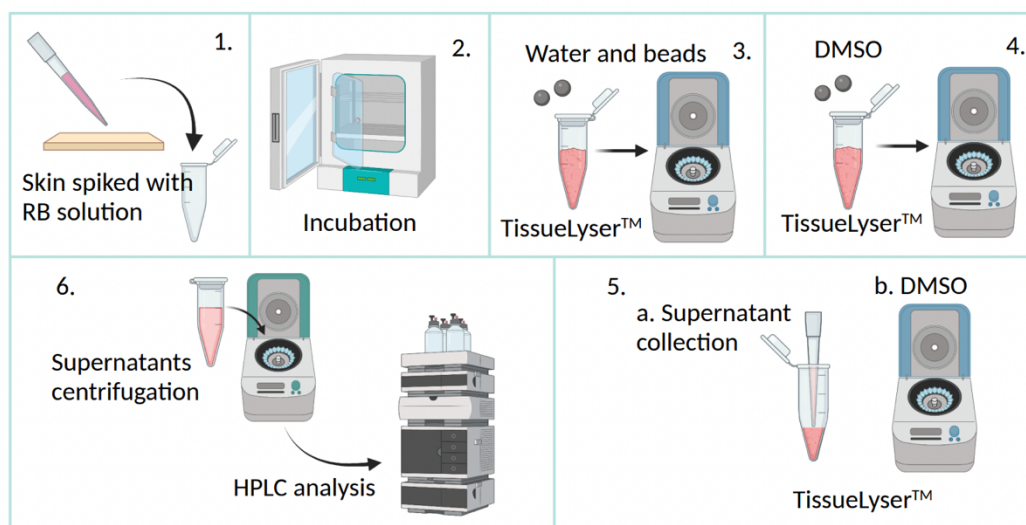


Fig. 2. Schematic illustration of the skin extraction recovery process.

The skin samples were then cut into fragments by a scissor and lysed at 50 Hz for 15 min by a Qiagen TissueLyser™ LT (UK Quiagen Ltd, Manchester, UK), following the addition of deionised water (0.5 mL) and two stainless steel beads (diameter=0.5 cm) (Qiagen, Hilden, Germany) [56,57]. Next, 1 mL of DMSO as extraction solvent was added [11], and samples were treated for a further 15 min (50 Hz): this step was repeated twice to ensure maximal RB extraction from the skin. The samples were then diluted in PBS, centrifuged at 14,000 rpm for 15 min (Sigma Laborzentrifugen

GmbH, Osterode am Harz, Germany) to precipitate skin components, and the supernatants were meticulously collected and quantified by HPLC using the HPLC-FLD. Results were compared with those obtained from the control group consisting of the same weight of RB without skin, treated as above. The RB percentage skin extraction recovery (ER%) was expressed as the mean of the extraction recovery values obtained from the low, medium, and high RB amounts tested (equation 5).

$$ER(\%) = \frac{RB \text{ quantified in skin samples}}{RB \text{ quantified in control samples}} \times 100 \quad (5)$$

2.5 Preparation of RB aqueous solution, RB loaded cream and RB loaded transfersomes.

RB was here loaded in three different formulations: (i) an aqueous solution, (ii) a water-based cream and (iii) a transfersomes dispersion. RB aqueous solution (RB-S, RB = 2 mg/mL) was obtained by dissolving 10 mg of RB in 5 mL of ultrapure water; the solution was then vortexed for 30 seconds at 2500 rpm to ensure the complete solubilisation of the drug. A water-based cream based on the British Pharmacopoeia (aqueous cream BP) was employed to obtain the RB-loaded cream (RB-C, RB = 0.2 mg/mg). RB (20 mg) was solubilised in 80 mg of the cream by a DAC 150 FVZ SpeedMixer (High Wycombe, England) at 3500 rpm for 5 minutes. RB-loaded transfersome dispersion (RB-TF) (RB = 2 mg/mL) was already prepared and characterised by the same authorship [38]; the technique employed for the preparation was reverse-phase evaporation. Briefly, 20 mg of RB was solubilised in 10 mL of ultrapure water and separately, the lipid phase (142 mg of Lipoid S100, 26 mg of cholesterol, 14 µL of Span® 80) was dissolved in 5 mL of ethanol. The two phases were subsequently submitted to a sonication process (60 s at 50% ultrasound (US) amplitude) using an ultrasonic probe device (Davidson & Hardy Ltd cooperating with Fisher Scientific, Leicestershire, UK) to obtain a homogeneous dispersion. Afterwards, ethanol was evaporated by a rotary evaporator (Rotavapor, Buchi Labortechnik, Switzerland) under vacuum at 50 °C. The resultant formulation was conserved for 1 h at room temperature in dark conditions, and six additional sonication cycles (10 s of 50% US amplitude / 20 s break) were finally applied.

2.6 Ex vivo skin permeation and deposition study

An *ex vivo* permeation and deposition study were performed across dermatomed neonatal porcine skin, testing RB-S, RB-C and RB-TF. The skin was obtained and prepared as reported above in section 2.5.

The study, conducted under infinite dose settings, employed Franz cells (Permergear, Hellertown PA, USA) with a 1.77 cm² orifice of an effective diffusion area of 0.36 cm² and 12 mL receptor volume. The system's temperature was thermally regulated at 37 ± 1°C to provide a skin surface temperature of 32 ± 1°C at the skin surface [58]. First, the receiver compartment was filled with 12 mL of degassed PBS pH 7.4 as the receiver medium; then, the skin was sandwiched between the donor and receiver compartment, with the subcutaneous side facing the receiver compartment. The skin was allowed to equilibrate for 30 minutes before experimenting [59].

The three formulations were applied separately on the top of the skin surface: 100 µL of RB-S, 100 µL of RB-TF dispersion, and 10 mg of RB-C. The donor and the sampling ports were sealed to minimise evaporation and contamination; the magnetic stirrers were set at 600 rpm to homogenise the receiver medium during the deposition study, which lasted 24 hours (figure 3).

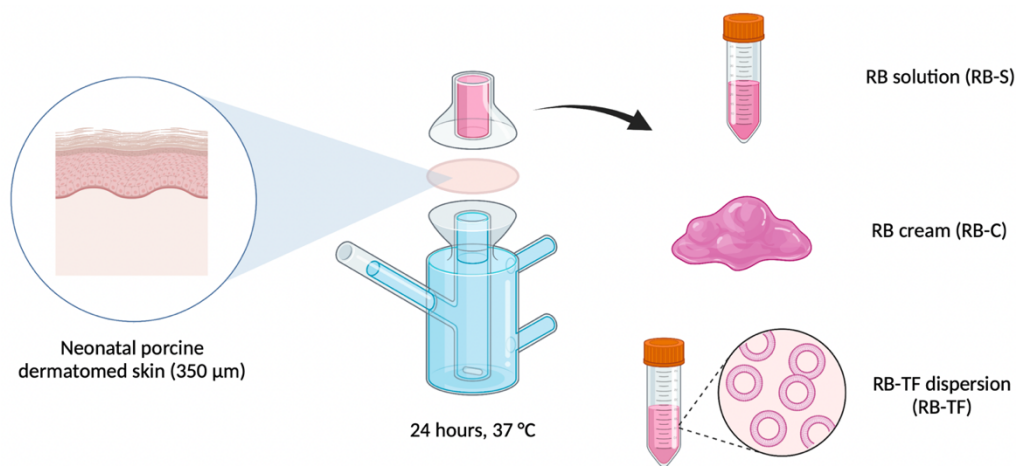


Fig. 3. Schematic representation of the set-up employed for the ex vivo permeation and deposition study.

Finally, both skin and receiver mediums were collected to quantify the RB amount deposited into the skin and the amount permeated. Any excess formulation from the solution, cream and dispersion on the skin surface was first wiped using wet tissue paper before processing the skin sample. The receiver medium was centrifuged, the supernatant was injected in the HPLC, and skin samples were processed as previously reported in section 2.5 before the quantification analysis. The amount of RB permeated and deposited into the skin was estimated by referring to the calibration curve obtained by the HPLC-FLD method. To quantify the amount of RB deposited into the skin, the ER was considered as well, according to equation 6:

$$RB \text{ deposited in the skin} = \frac{RB \text{ quantified in skin samples}}{ER} \quad (6)$$

2.7 Multiphoton microscopy investigation

The deposition of RB-S, RB-C and RB-TF in the dermatomed skin was visually examined at the end of the *ex vivo* deposition study by multiphoton microscopy. Micrographs were obtained using a Leica TCS SP8 multiphoton scanning microscope (Leica Microsystems Ltd, Milton Keynes, UK) set with an upright DM6 microscope body and a motorised stage. The Leica Application Suite X software (3.5.7.23225) was employed to process the images. Samples were excited with 549 nm laser lines from the Mai Tai Deep See Mode-Locked laser system (Newport- Spectra-Physics, UK). Fluorescence emission was detected via HyD GaAsP-spectral detectors for Rose Bengal dye between 557 nm and 660 nm. A water immersion objective HC FLUOTAR L 25x with 0.95 Numerical Aperture or dry objectives HC PL APO 10X/0.40NA or HC PL FLUOTAR L 40X0.60NA was employed to acquire images as suitable. Micrographs were acquired at 1024x1024 pixel resolution format and a scanner speed of 400 Hz. Image analysis was done using the Leica Application Suite X software (3.7.020979).

2.8 Examination of the fluorescence spectra

The fluorescence spectra of RB-S, RB-C, RB-TF, skin spiked with RB formulations, and blank skin was carried out as previously reported by [46]. The spectra were recorded with a fluorescence spectrophotometer (Hitachi, Honshu, Japan), exciting each sample at 549 nm. Results were finally processed with GraphPad Prism® 9.4.1. In order to analyse RB-S, RB-C and RB-TF, the samples were diluted with PBS previously filtered (regenerated cellulose syringe filter, pore size: 0.20 µm, filter size: 15 mm, Albet LabScience, Dassel, Germany) to achieve a 10 µg/mL RB concentration; the auto zero was performed with blank PBS. Regarding the analysis of skin spiked with RB-loaded formulations, the skin was first spiked with RB-S, RB-C and RB-TF separately and subsequently processed as reported in section 2.5. Blank skin, submitted to the same treatment, was examined and used to perform the auto zero.

2.9 Statistical analysis

Data were statistically analysed using GraphPad Prism® 9.4.1 (GraphPad Software, San Diego, California, USA). Multiple unpaired t-tests analysed accuracy, precision, and skin extraction recovery. Two-way ANOVA was used to determine whether the RB permeated across and deposited into the skin. A p value < 0.05 was used to indicate statistically significant differences in all cases.

3. Results and Discussion

3.1 HPLC analytical method

RB is a molecule with several applications in dermatology, and an analytical procedure to determine the molecule's content within the skin upon delivery is highly demanded. The quantitative analysis of a drug in such a type of matrix is challenging because of the low expected concentrations, small sample volumes and the intrinsic structure of the skin itself. Indeed, the skin is considered a hard tissue, meaning a more robust extraction procedure and sample preparation are required to accomplish an accurate analysis relative to soft or tough tissues. Moreover, the endogenous components extracted from the biological matrix during sample preparation can easily interfere with the detection of the analyte. This is further complicated by the log P of RB (0.59), which causes the molecule to exhibit a natural affinity to skin components, making the extraction and, consequently, the quantification harder [60,61].

3.2 Analytical method optimisation

The chromatographic conditions used as starting lines were those proposed by Mannan et al. to quantify RB from surgical strips [40]. This method was accurate, rapid, and specific, but it was not sensitive enough for the purpose of our work, as it reported an LLOQ of 3 µg/mL and LOD of 1 µg/mL. To improve the sensitivity, we first tried to change the excitation wavelength maintaining the UV-vis detection. Secondly, we changed the technique of detection from UV-vis to FLD. The three HPLC methods are summarised in table 1.

Table 1. Chromatographic conditions used to develop the method for the quantification of RB in the skin.

<i>Condition</i>	<i>Starting method*</i>	<i>UV-vis HPLC</i>	<i>FLD-HPLC</i>
Mobile phase	MeOH and Buffer pH 8 ¹ 50/50 v/v	MeOH and Buffer pH 8 ² 60/40 v/v	MeOH and Buffer pH 8 ² 60/40 v/v
Pump mode	Isocratic	Isocratic	Isocratic
Diluent	Buffer pH 8**	PBS pH 7.4	PBS pH 7.4
Column	C-18 Chromosil 100-5 µm (250 x 4.6 mm)	C-18Phenomenex SphereClone® (150 x 4.6 m)	C-18Phenomenex SphereClone® (150 x 4.6 m)
Column temperature	Ambient	Ambient	Ambient
Excitation wavelength	262 nm	549 nm	556 nm
Emission wavelength	-	-	573 nm

Injection volume	20 µL	40 µL	40 µL
Flow rate	1 mL/min	1 mL/min	1 mL/min
Run time	10 min	7	6
Retention time	2.69 min	3.1 min	4.1 min

*Mannan et al. [40].

¹ potassium phosphate dibasic adjusted with sodium hydroxide or phosphoric acid.

² ammonium acetate adjusted with sodium hydroxide.

Due to the extremely low water solubility of Rose Bengal (molecular formula C₂₀H₄Cl₄O₅), in this investigation, we employed the ionisable form RB disodium salt (solubility in water: 100 g/L). Aqueous solubility is a desirable property for a drug molecule as it facilitates the interaction with the pharmacological target. For this purpose, the poor solubility of potentially active molecules can be modified by strategies recognised in contemporary medicinal chemistry, including exploiting any potential ionisable centres. The General Solubility Equation (eq.7), used to predict the solubility for unionised molecules, can be modified to reflect the contribution of charges to solubility at any given pH by substituting log P for log D [62]:

$$\log S = -\log P - 0.01 * (M_{pt} - 25) + 0.05 \quad (7)$$

The increased ionisation reduces logD at given pH and increases the aqueous solubility. Since we used the ionisable form of RB, the mobile phase selection was pivotal to achieving an adequate ionisation degree [62]. The selected pH for the mobile phase was 8, as RB is entirely ionised and consequently solubilised (Fig 4b). The aqueous part of the mobile phase consisted of a phosphate buffer at pH 8.0, a value at which RB is entirely ionised, based on the Log D prediction shown in figure 4.

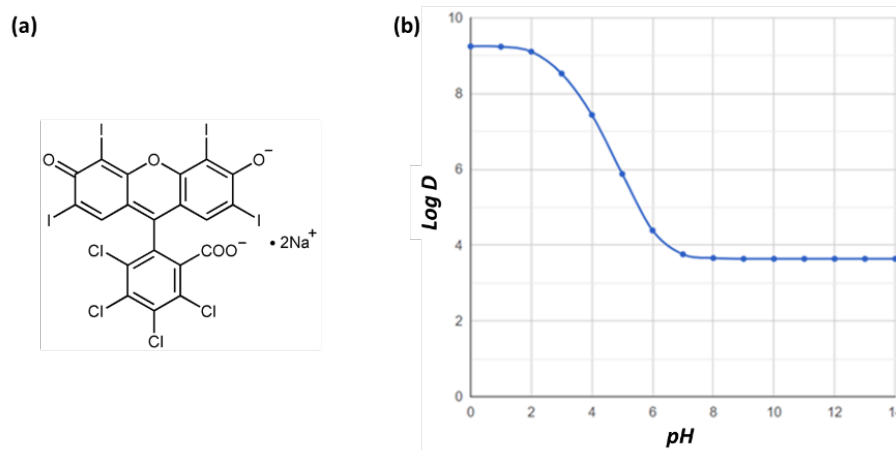


Fig. 4. (a) Chemical structure of RB disodium salt. (b) Log D of RB as a function of pH predicted using ChemAxon software.

The starting method employed the excitation wavelength of 262 nm for RB determination in surgical strips [40]. Unfortunately, at lower wavelengths, the likelihood of interfering peaks arising in the chromatogram increases, especially within biological matrices [63]. Therefore, we decided to shift this value to the maximum RB absorption wavelength, set at 549-562 nm [39]. The two methods' linearity, LOD and LLOQ, were calculated to identify the most suitable detector. Results are reported in Table 2, and the calibration curves are represented in figure 5.

Table 2. Properties of the calibration curve obtained using a fluorescence detector and UV detector to quantify RB with LOD and LOQ values ($n = 6$).

<i>Detector</i>	<i>Slope</i>	<i>Intercept</i>	<i>Linearity</i> ¹	<i>LOD</i> ²	<i>LLOQ</i> ²
UV-vis	190.5	16.84	1.0000	0.60	1.83
FLD	327.3	-58.28	0.9999	0.17	0.54

¹ Linearity is expressed as R squared.

² LOD and LLOQ are expressed in $\mu\text{g/mL}$.

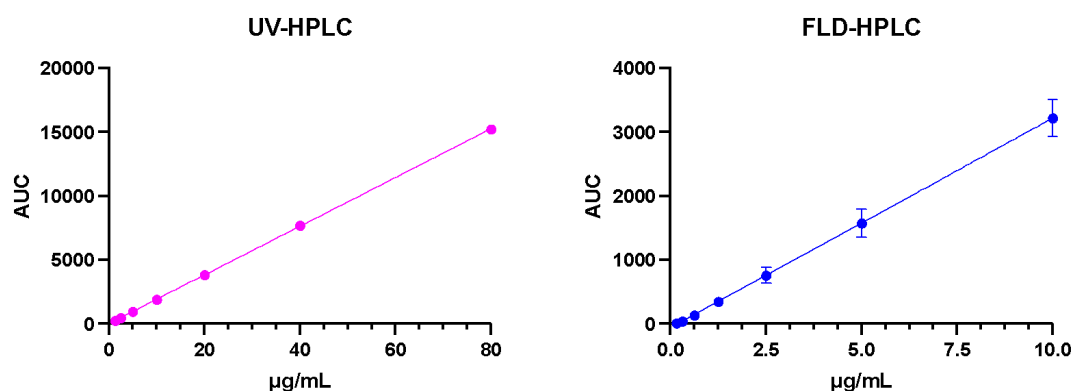


Fig. 5. Calibration curves of RB obtained with UV-HPLC and FLD-HPLC detection (means $\pm$ SD, $n=6$).

The data show that the calibration curves displayed a linear response with a regression coefficient (R^2) ≥ 0.9999 over the concentration range evaluated. On the other hand, the LLOQ was found to be 0.54 $\mu\text{g/mL}$ for the HPLC-FD method, which was more sensitive than the HPLC-UV method, which had an LLOQ of 1.83 $\mu\text{g/mL}$. The superior sensitivity obtained from HPLC-FD is attributed to the greater sensitivity conferred by fluorescent detectors compared to UV detectors [64]. Moreover, it is widely known that most polycyclic aromatic hydrocarbons, such as RB, can easily be observed via fluorescence-based techniques. The delocalised electrons in the aromatic rings can easily be excited. In addition, the stiff structure of polycyclic rings does not allow for efficient vibrational relaxation, enabling sufficient time for the emitted fluorescence to be detected [65]. Considering these outcomes, fluorescence detection was finally selected as the leader RB determination method and further evaluated.

3.3 Specificity of the HPLC-FLD method

The specificity of the HPLC-FLD method was determined by evaluating the chromatograms of RB in the diluent and the presence of the skin matrix's interferences. As reported in figure 6, no interfering peaks were detected, suggesting the specificity of the current method. The fluorescent detector specificity prevents co-eluting peaks that typically arise when quantifying analytes from the biological matrix using HPLC-UV. Such advantage is attributed to the low concentration of naturally fluorescent compounds found within biological tissue. However, a change in the RB retention time in the presence of skin has been denoted, shifting from 4.1 min in PBS to 3.5 min in the skin. In this regard, it has already been described that the complex composition of the skin matrix provides samples that carry on a considerable diversity of endogenous substances, in contrast to the more homogeneous plasma samples [61]. This phenomenon, coupled with the

known RB affinity for skin components, turned herein into a slight variation of the retention time without affecting the specificity of the method for the analyte.

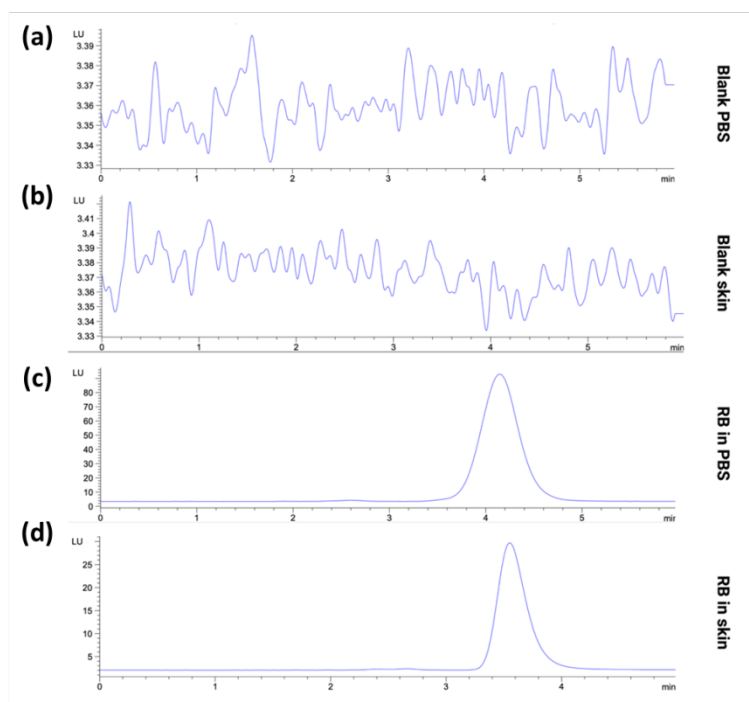


Fig. 6. Representative HPLC-FD chromatogram of (a) blank PBS, (b) blank skin extract, (c) RB in PBS (10 µg/mL), and (d) RB from skin extract (5 µg/mL).

3.4 Accuracy and precision of the FLD-HPLC method

Accuracy and precision were evaluated over one day (intra-day variability) and three consecutive days (inter-day variability); values are shown in table 3. No statistically significant difference was observed between the theoretical and experimental concentrations (p -value > 0.05). The values of RE% for all QC samples were in the range of 1.17 – 2.42%. The precision, which indicates the method’s repeatability, exhibited an RSD% value from 0.98 to 3.06%, within the concentration range tested. The results confirmed that the new analytical method is accurate and precise since the values obtained were lower than the acceptable value of 15%; hence, they are under the recommendations for bioanalytical methods [60,66,67]. Based on these results, it can be assumed that the current approach is potentially appropriate for quantifying RB deposited into skin samples.

Table 3. Intra-day and inter-day accuracy and precision of RB (means $\pm$ SD, $n = 6$).

	<i>Theor. concentration (µg/mL)</i>	<i>Exp. concentration (µg/mL)</i>	<i>Precision (RSD%)</i>	<i>Accuracy (RE%)</i>

Intra-day	8.00	7.89 ± 0.08	1.02	-1.33
	4.00	3.95 ± 0.07	1.91	-1.26
	1.00	0.98 ± 0.03	2.65	-2.42
Inter-day	8.00	7.90 ± 0.08	0.98	-1.17
	4.00	3.93 ± 0.10	2.57	-1.84
	1.00	0.98 ± 0.03	3.06	-1.95

3.5 Carry-over

The signal of RB possibly interferes with the measurement of the blank solution; therefore, the carry-over evaluation was performed. In order to evaluate this, a high concentration of RB solution (50 µg/mL) was injected, followed by a blank sample. The result obtained was that the peak of RB was not detected in the sequence of blank samples. Accordingly, it indicates no carry-over effect in the developed HPLC method.

3.6 Dilution integrity

The dilution integrity was assessed by monitoring the RB concentration's consistency upon diluting 5 and 10 times lower than the stock solution. As presented in Table 4, the results showed that the RSD and RE of both dilutions were in the range of 0.98-1.53% and 4.22-4.70, respectively. This indicates that the dilution integrity is reliable for the developed HPLC method, as the values of RSD and RE were in the acceptable range, which is less than 15%.

Table 4. Dilution integrity developed HPLC method for RB (means ± SD, n = 3).

Dilution	Recovery (%)	Precision (RSD%)	Accuracy (RE%)
5 times	104.70 ± 1.02	0.98	4.70
10 times	104.22 ± 1.60	1.53	4.22

3.7 Skin extraction recovery

A bead homogenisation approach has been employed to extract RB from the skin. This method breaks down the skin tissue by utilising stainless-steel grinding balls combined with a lysing solvent to extract RB. Through the high-velocity collision provided by the TissueLyser®, the beads grind and disrupt the cell membranes releasing the intracellular fluid [61]. The supernatant of the resultant homogeneous mix has been centrifuged and used to quantify RB. The bead homogenisation

technique has already been applied for porcine tissues and suits all skin samples. Herein, dermatomed skin, with a thickness of 350 μm , was selected to limit the influence of the tissue thickness variability on the method's validity [61,68]. It is important to remember that the extraction of RB was performed on the entire dermatomed skin without separating the epidermis from the dermis. Herein, DMSO was employed as lysing solvent, and it is recurrently used in biological studies because the solvent quickly penetrates and diffuses through biological membranes [69]. Furthermore, DMSO can dissolve the skin lipids or denature skin proteins. It is a very efficient solvent due to its intrinsic amphiphilic properties arising from its hydrophilic sulfoxide group and two hydrophobic methyl groups [70]. Considering the amphiphilic nature of RB, DMSO was considered the best candidate.

RB's extraction recovery (ER%) was determined by comparing the concentration of different spiked skin samples to the standard solution as a control to understand if the technique mentioned above could provide reliable results. The results are displayed in table 5. The RB recovered from dermatomed skin varied from $95.51 \pm 2.49\%$ to $97.62 \pm 5.89\%$; the RSD% values fall within the range of 2.61%-6.03%. No statistically significant difference between the RB concentration added and the concentration recovered was observed (p value > 0.05). The recovery was within the $100 \pm 10\%$ limit reported in the OECD guideline, and RSD values are below the maximum limit of 15%. These suggest that the extraction procedure was highly efficient, consistent, precise, and reproducible [71–73].

Table 5. Skin extraction recoveries of RB from dermatomed neonatal porcine skin (means $\pm$ SD, $n = 4$).

RB concentration added ($\mu\text{g/mL}$)	ER% $\pm$ SD	RSD%
1	97.18 ± 2.33	2.40
4	98.05 ± 8.79	8.96
8	100.82 ± 4.53	4.50

3.8 Ex vivo skin permeation and deposition study.

The analytical and extraction methods were then used to quantify the amount of RB permeated into and across dermatomed porcine skin following a 24-hour permeation study.

The intact SC is the primary barrier to drug penetration [1]. To overcome this limitation, a drug that acts within the skin can be formulated in several topical delivery systems, from the most conventional to the most advanced. Conventional formulations include solutions, ointment, gels, or

creams, whereas newer drug delivery systems utilise nanoparticles and mechanical approaches that reversibly disrupt the SC [74–76]. Despite the range of approaches employed to enhance the delivery of therapeutics into the skin, nano-systems, such as liposomes, remain the most employed in clinics. With these assumptions, three types of topical formulations were tested herein: RB aqueous solution (RB-S), RB water-based cream (RB-C) and RB transfersomal dispersion (RB-TF). Figure 7 reports the percentage of RB deposited within the skin and the receiver compartment.

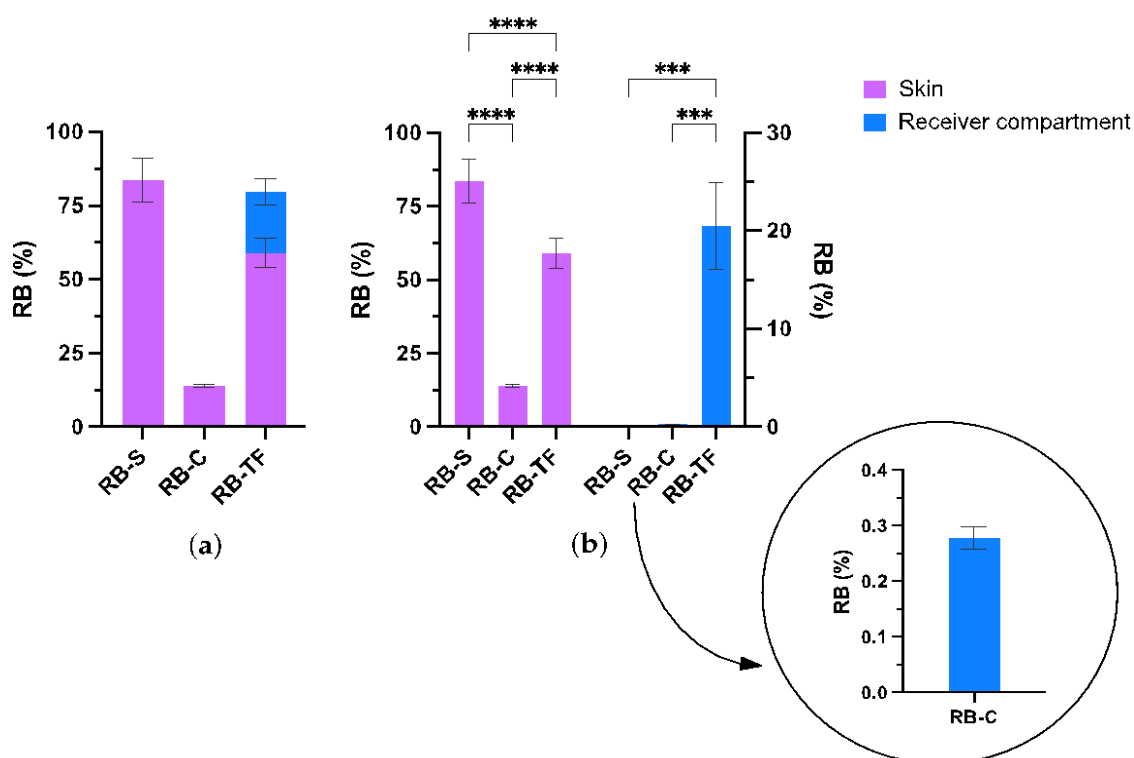


Fig. 7. Amount of RB recovered after 24 hours of ex vivo deposition study across dermatomed porcine skin (n=3). (a) Illustration of the total amount of RB detected at the end of the study. (b) Focus on the amount of RB detected within the skin layers and in the receptor compartment. **** p value < 0.001; *** p value < 0.01.

The percentage of RB deposited into the skin was 83.57 ± 7.52 in the case of RB-S, 13.86 ± 0.48 in the case of RB-C, and 59.07 ± 5.06 per RB-TF. At the same time, the percentage of RB permeated into the receiver compartment was 0.28 ± 0.02 and 20.53 ± 4.41 in the case of RB-C and RB-TF, respectively. In contrast, RB did not permeate across the skin when delivered by RB-S.

The data reported agreeing with the characteristics of the RB-loaded delivery system prepared. The poor permeation profile for RB-S may be attributed to the physicochemical properties of the drug. The stratum corneum (SC) is the main barrier to the penetration of external agents. This is especially true for molecules exceeding 500 Da possessing anionic or cationic charges. RB disodium salt is a highly water-soluble amphiphilic drug with two anionic charges in the solution and a molecular weight of 1017.64 g/mol. RB aqueous solution (RB-S) did not permeate across the skin,

and most of it was deposited into the skin tissue. In this investigation, no physical separation of epidermis and dermis was performed; however, RB is likely to accumulate in the epidermal layer without significantly reaching the dermis, as demonstrated in our previous work [30,38]. Incorporating RB into the aqueous cream (RB-C) led to the lowest deposition and permeation efficacy compared to RB-S and RB-TF. Aqueous creams are oil-in-water emulsions in which the water-soluble drug typically dissolves in the water phase [74]. The cream is composed of emulsifying ointment (30% w/w), soft white paraffin (15% w/w), liquid paraffin (6% w/w) and purified water which is the typical composition for aqueous cream BP [77]. Considering RB's affinity towards lipids, it is likely that RB may interact with the excipients present within the cream, cetostearyl alcohol, and sodium lauryl sulphate even post-application [46]. Aqueous cream BP is commonly used to manage dry skin by forming an oily layer on the top of the skin, decreasing the skin's water loss via simple occlusion [78]. Based on this, we hypothesised that the oily phase of RB-C deposited on the top of the skin is responsible for the RB detected in the skin layers. This protective film which serves as an occlusive layer, also mitigates the permeation of RB from the formulation into and across the skin following application.

Formulating RB into TF significantly improved the transdermal delivery of RB. RB-TF proved to be small unilamellar vesicles (SUV) with an average size of 62.91 ± 6.28 nm, a PDI of 0.271 ± 0.045 , and a zeta potential of -38.47 ± 0.20 mV [38]. In this investigation, the amount of RB deposited into the skin layers following the application of RB-TF was considerably lower than RB-S (p value < 0.001), and the amount permeating the whole skin was significantly higher than both RB-S and RB-C (p value < 0.01). In this regard, TF is liposome-like vesicles first developed by Gregor Cevc to improve the migration through the skin compared to their predecessors, the liposomes. In contrast to the rigidity of conventional liposomes, the presence of the surfactant within TF's structure provides flexibility to the vesicle and allows TF to squeeze and pass-through skin pores without losing their integrity while being driven by the transdermal hydration gradient, ultimately improving the delivery efficiency [79].

These results remarked that the HPLC-FD method developed in this work could effectively quantify and elucidate the permeation profile RB across the skin from different delivery systems. The sensitivity conferred by the method developed would enable formulators to evaluate which formulation can exhibit the highest delivery efficiency. Such a decision would be paramount in moving the formulation into preclinical and clinical studies.

3.9 Multiphoton microscopy investigation.

To further evaluate the performance of the RB-loaded dermatological formulations, we investigated RB's distribution within the dermatomed skin by multiphoton microscopy. Multiphoton microscopy, such as other fluorescence-based microscopical techniques, utilises the intrinsic fluorescence properties of some molecules in order to investigate the delivery of those molecules within the skin's layers [49]. Herein, we present the micrographs of the skin samples at the end of 24 hours of the permeation experiment (figure 8). Two types of images are shown for each sample (RB-S, RB-C, RB-TF): the first type was acquired as a unidimensional picture of the skin from the top view of the SC, and the second type is a 3D-visualisation obtained by multiple horizontal scanning of the dermatomed skin enabling us to see entire tissue thickness. The pictures illustrate only the RB distribution without explicitly showing the skin, which was subtracted by the instrument software.

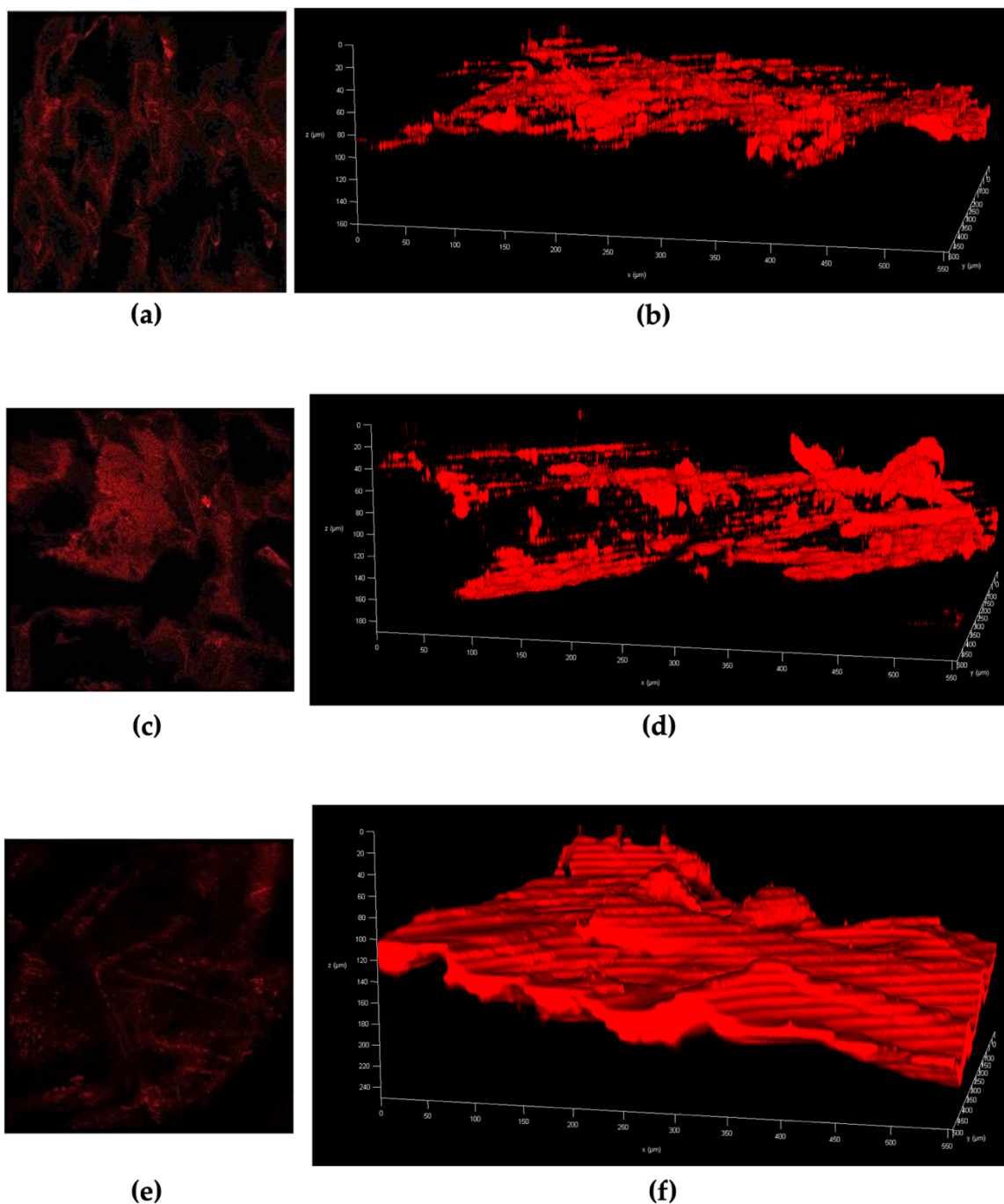


Fig. 8. Micrographs of dermatomed skin samples at the end of 24-hours permeation study acquired by multiphoton microscopy. (a) RB solution (RB-S), stratum corneum; (b) RB-S, 3D-visualisation; (c) RB-loaded Aqueous Cream BP (RB-C), stratum corneum; (d) RB-C, 3D-visualisation; (e) RB loaded TF dispersion (RB-TF), stratum corneum; (f) RB-TF, 3D-visualisation.

The overall thickness of the dermatomed porcine skin is $350 \mu\text{m}$ of which $83.7 \pm 16.6 \mu\text{m}$ of this tissue constitute the epidermis while the SC represents $14.8 \pm 4.8 \mu\text{m}$. Lastly, the remaining layers of the dermatomed skin are made up of viable epidermis [38]. Regarding RB-S, we hypothesised that the RB preferentially accumulates within the epidermis with minimal to no transdermal permeation. Figure 7A shows that RB was visible in the most superficial layers of the skin, and figure

7B reports an intense yet not entirely homogeneous distribution up to 80-100 μm depth. This depth also reflects the thickness of the epidermis. RB-C was the formulation with the deposition of RB into the skin with very low transdermal permeation. Figure 7C reports a high and non-uniform distribution of RB-C in the outermost skin layer. We previously suggested that the oily phase of RB-C was likely to form a film on the skin surface, acting as a barrier to the permeation of RB loaded in the water phase. Furthermore, considering that the excipients of the oily phase tend to emulsify with the skin components [74], the RB loaded in the oily phase may diffuse through the skin into the dermis and thus result in some degree, albeit minimal, transdermal permeation. This is corroborated by the data presented in figure 7D, showing that the RB staining is less consistent than in figures 7B and 7F; its distribution was primarily limited in the range of 20 – 160 μm depth, reaching the dermis. Finally, RB-TF was found to enhance the deposition of RB into the skin and augment the compound's permeation into the receptor compartment. Figure 7E proves that RB is visible in the most superficial layer, even if the staining is not as intense as in the case of RB-S. On the other side, figure 7F shows intense and uniform staining, mainly ranging between 40 – 220 μm depth; in some points, RB is also detectable upper 40 μm depth. Hence, RB-TF was deposited primarily on the lower epidermis and dermis, which ultimately resulted in the delivery of the compound into the receiver fluid. This data, in tandem with results from the newly developed HPLC-FD, demonstrates the utility of TF as a novel and potentially versatile dermal and transdermal drug delivery system.

The results discussed here align with the *ex vivo* permeation, and deposition study previously commented on, highlighting the suitability of multiphoton microscopy in investigating drug distribution through the skin. In addition, the imaging capability of this technique elegantly complements the quantitative data presented from the HPLC analysis.

3.10 Examination of the fluorescence spectra.

The investigation of the fluorescence emission spectra of RB was carried out to evaluate RB's tendency to interact with the components of various environments, e.g., transfersomal dispersion, aqueous cream, and skin (figure 9). The emission spectra recorded highlight how RB's maximum emission wavelength (λ_{max}) and fluorescence intensity (FI) strongly depend on the composition of the environment in which RB is added. Regarding the spectra of the formulation themselves, RB-S and RB-C showed the same λ_{max} (566.5 nm) and similar FI, 260.1 and 227.4 for RB-S and RB-C, respectively. In the case of RB-TF, the λ_{max} was recorded at 551.5 nm with a FI of 480. The similarity of the RB-S and RB-C spectra can be attributed to the water-based nature of the RB-C. On the other

side, RB loading into TF led to a high increase in the FI compared to RB-S and RB-C. The enhanced intensity indicates that RB intercalated through the lipid bilayer mainly in its monomeric form but not as a dimer, effectively limiting the formation of RB aggregates [46]. For this purpose, the aggregation of RB monomers into dimers has been detected in RB aqueous solutions at a concentration above 2.0×10^{-6} M, affecting its excitation and emission spectra [80]. Different outcomes were reported concerning skin samples spiked with RB formulations, as the variances in the emission spectra were much less pronounced. In this case, the values recorded were a λ_{max} 552.5 nm and FI 301.8 for RB-S, a λ_{max} 560 nm and FI 329.7 for RB-C and a λ_{max} 588 nm with a FI 403.4 in the case of RB-TF. We previously reported that DMSO dissolves skin lipids and, simultaneously, the lipids constituting the formulations tested [69]. The skin samples, spiked with RB-loaded formulations, were all submitted to the same extraction process described in section 2.5, which employed DMSO as a lysing solvent. We hypothesised that, in this way, the samples analysed might be similar in composition and molecular bonds, exhibiting similar emission spectra. Nevertheless, a difference can still be noticed since the lipid-based formulation, RB-TF, showed a higher intensity signal.

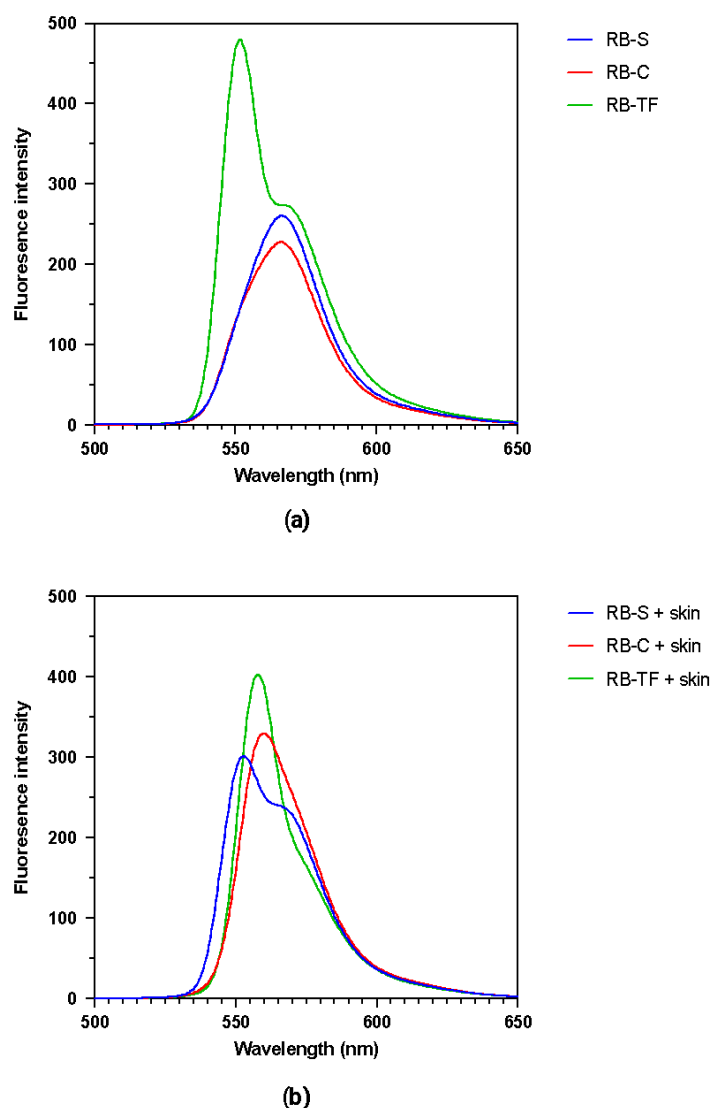


Fig. 9. Fluorescence spectra of RB in different environments. (a) RB solution (RB-S), RB loaded Aqueous Cream BP (RB-C) and RB-TF dispersion. Samples were diluted with PBS, and the autozero was performed using blank PBS. (b) Samples obtained from skin spiked with RB-S, RB-C and RB-TF. The auto zero was performed using a sample obtained from blank skin.

4. Conclusions

The study presented describes the fluorescence-based techniques that might be used to determine RB qualitatively and quantitatively in the skin. Herein, a sensitive analytical method was validated for quantifying RB using the HPLC technique coupled with a fluorescence detector for the first time. The analytical method was validated by considering the standards proposed by the ICH, FDA and EMA guidelines, and it was demonstrated to be specific, precise, and accurate. Following topical application, the HPLC method successfully assessed RB's *ex vivo* from three different delivery systems. In terms of qualitative determination, it was shown that multiphoton microscopy allows

for tracking the RB distribution within the skin tissue, and the analysis of the fluorescence spectra provides crucial complementary information concerning the behaviour of RB under different milieus. Such an approach may play an essential role in preclinical RB formulation screening, representing an effective tool for developing a successful skin-targeted delivery system.

CRedit authorship contribution statement

Sara Demartis and Qonita Kurnia Anjani: Conceptualisation, Sara Demartis and Qonita Kurnia Anjani: methodology, Sara Demartis, Qonita Kurnia Anjani and Fabiana Volpe-Zanutto: software; Sara Demartis and Qonita Kurnia Anjani: validation; Sara Demartis, Qonita Kurnia Anjani, Fabiana Volpe-Zanutto and HuanHuan Li: formal analysis, Sara Demartis and Qonita Kurnia Anjani: investigation; Ryan F. Donnelly: resources, Sara Demartis, Qonita Kurnia Anjani and HuanHuan Li: data curation, Sara Demartis, Qonita Kurnia Anjani, and Akhmal Bin Sabri: writing—original draft preparation Sara Demartis, Qonita Kurnia Anjani, and Akhmal Bin Sabri: writing—review and editing, Sara Demartis, Qonita Kurnia Anjani Ryan F. Donnelly and Elisabetta Gavini; visualisation, Ryan F. Donnelly and Elisabetta Gavini: supervision and project administration.

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Research Article

Trilayer dissolving polymeric microneedle array loading Rose Bengal transfersomes as a novel adjuvant in early-stage cutaneous melanoma management.

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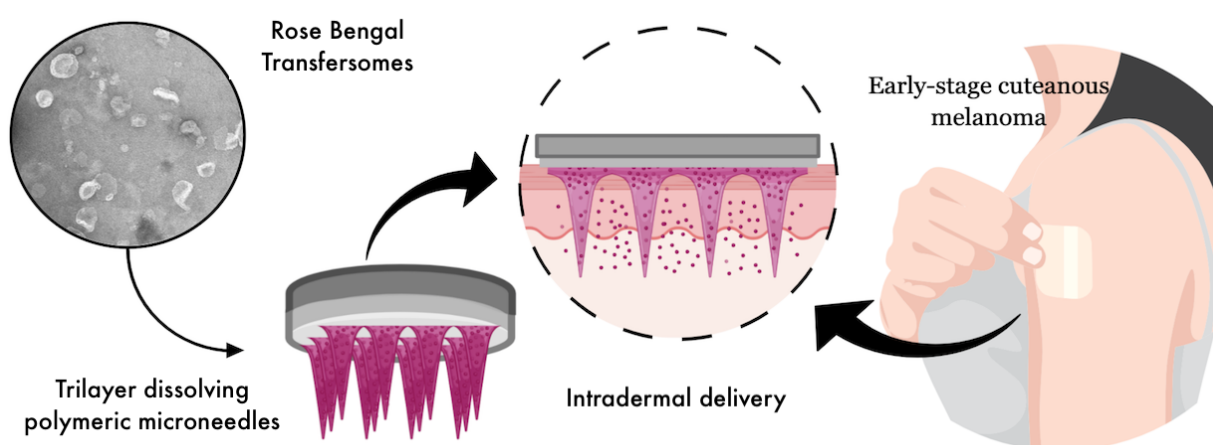
The current experimental section describes the development of a Dissolving Microneedles Array Patch loading Rose Bengal Transfersomes. This investigation was performed over the PhD's second and third Academic Years, during the visiting to the Prof. Ryan Donnelly's lab (School of Pharmacy Queen's University Belfast). Lastly, the investigation was published as an Original Research Article titled "*Trilayer dissolving polymeric microneedle array loading Rose Bengal transfersomes as a novel adjuvant in early-stage cutaneous melanoma management.*" in the peer-reviewed and indexed Journal "International Journal of Pharmaceutics (2021 Journal Impact Factor (JIF): 6.510; JIF Quartile: Q1 in the category Pharmacology and Pharmacy).

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Abstract

Melanoma remains a global concern, but current therapies present critical limitations pointing out the urgent need for novel strategies. Among these, the cutaneous delivery of drugs selectively damaging cancer cells is highly attractive. Rose Bengal (RB) is a dye exhibiting selective cytotoxicity towards melanoma, but the high water solubility and low permeability hinder its therapeutic potential. We previously developed RB-loaded transfersomes (RBTF) to mediate the RB dermal delivery; however, a platform efficiently delivering RBTF in the deepest strata is essential for a successful therapeutic activity. In this regard, dissolving microneedles release the encapsulated cargo up to the dermis, painlessly piercing the outmost skin layers. Therefore, herein we developed and characterised a trilayer dissolving microneedle array (RBTF-TDMNs) loading RBTF to maximise RBTF intradermal delivery in melanoma management. RBTF-TDMNs were proven strong enough to pierce excised porcine skin and rapidly dissolve and deposit RBTF intradermally while maintaining their physicochemical properties. Also, 3D visualisation of the system itself and while penetrating the skin was performed by multi-photon microscopy. Finally, a dermatokinetic study showed that RBTF-TDMNs offered unique delivery efficiency advantages compared to RBTF dispersion and free drug-loaded TDMNs. The proposed RBTF-TDMNs represent a valuable potential adjuvant tool for the topical management of melanoma.

Keywords: Rose Bengal; Water-soluble drug; Transfersome; Microneedle; Melanoma; Intradermal Delivery.



1. Introduction

Cutaneous melanoma is one of the most aggressive forms of skin cancer and one of the leading causes of cancer-related mortality due to its metastatic power. From 1990 to 2019, the incidence of melanoma increased by 170% to 289,950 and death increased by 90% to 62,840 globally (Li et al., 2022a). Most patients with newly diagnosed melanoma suffer from an early-stage disease still localised to the skin (American Cancer Society TM, 2019). The majority of those cases have a good prognosis and are usually treated by the surgical excision, which removes the primary tumour with adequate margins to ensure the best chances of long-term survival [2,3]. The historically supported surgical margins to minimise the possibility of late recurrence are 5 cm, measured from the edge of the lesion during surgery. However, removing such a wide tissue section is not convenient when the disease affects anatomical locations such as hands, feet, head, neck and the anogenital area. Indeed, apart from aesthetic disfiguring, this procedure implies a complex reconstruction, long-term recovery and temporal disability, with increased morbidity in the previously mentioned anatomical area [2,3]. Non-surgical therapeutic approaches include chemotherapy, immunotherapy, radiotherapy and photodynamic therapy, along with the limitations of induced resistance to immuno-chemotherapeutics or intralesional treatment and side effects generally caused by the lack of selectivity [4]. Hence, surgical resection remains the primary option; nevertheless, there is an urgent need to reduce the surgical margins to improve the patient's recovery and compliance without posing a risk of a late recurrence. Considering the localisation of melanoma at an early stage, malignant cells are easily accessible from the skin's surface, which can be potentially used as a localised delivery route for immuno-chemotherapeutics, avoiding systemic administration and related disadvantages [5]. However, the stratum corneum (SC) is a widely documented barrier to solute penetration, restraining topical and transdermal bioavailability of the active pharmaceutical ingredients (APIs) [6,7].

Rose Bengal (RB) is a dye mainly known for its colouring and photosensitising properties [8]. The intrinsic cytotoxic of RB was discovered in 1986 [9]; since then, its anticancer activity has been tested, demonstrating its selective and effective tumour toxicity on melanoma, breast cancer, ovarian carcinoma, and colon and gastric cancers. [10]. In-vitro studies proved that RB induced death in melanoma cell lines secondary to releasing proteases, preferentially entering cell lysosomes, and sparing healthy cells [11,12]; Based on these outcomes, the intralesional injection

of 10% Rose Bengal disodium solution (PV-10™) was tested in clinical trials to treat the metastatic disease with promising results (ClinicalTrials.gov Identifiers: NCT00521053, NCT00219843). In this regard, a study explored the intralesional injection of PV-10™ in melanoma-bearing mice and elucidated a second systemic RB mechanism of action due to the onset of a tumour-specific immune response [13]. In the view of managing melanoma at its early stages, a topical application of RB is undoubtedly preferable to the current invasive route of administration. Nevertheless, the most substantial challenge in delivering RB through the cutaneous way is related to its high molecular weight (1017.64 g/mol), water solubility (100 g/L) and anionic nature. These characteristics limit the RB permeation through the biological barriers, including SC, as well as the cellular uptake and, consequently, its therapeutic potential [10]. To obviate this issue, we previously formulated RB into green and biocompatible flexible lipid nanovesicles, transfersomes (TF) [14]. The in-vitro release study highlighted that RB-loaded TF (RBTF) were able to release RB in a controlled manner compared to RB aqueous solution; furthermore, the ex-vivo epidermis permeation study showed that RBTF significantly improved RB permeation through the epidermis. Also, the RBTF blocked the proliferation of melanoma cells in-vitro in a more effective way compared to the free drug in solution; hence, loading RB in TF is critical to enhancing cellular uptake and therapeutic efficiency. However, its current pharmaceutical form, a liquid dispersion, does not have the required consistency for topical application, and the RB dosage and release cannot be suitably controlled. For this purpose, semisolid or solid-like formulations are the preferred vehicles as they stay in situ and deliver drugs in a better-controlled manner [15]. This scenario offers an exciting prospect to explore a novel platform that efficiently delivers RBTF while maintaining its physicochemical properties to preserve the advantages provided by nanotechnology.

For the last decades, microneedle (MN) arrays have actively been investigated as a physical enhancement of cutaneous drug delivery as they disrupt the skin barrier innovating the approach to treating skin diseases [16]. Dissolving MN arrays have gained much attention for their biocompatibility, excellent biodegradability, and high loading capacity of APIs (Li et al., 2022b; Paredes et al., 2021). Fabricated with soluble polymers, dissolving MNs gradually dissolve in the tissue fluid upon skin application by creating temporary aqueous microchannels, consequently releasing the encapsulated cargo in a controlled manner with precise drug dosage. The advantages of using dissolving MNs rely on a minimally invasive device that is easily self-administrable to the skin without causing any discomfort or bleeding, exposing the deepest skin strata to the cargo depot following a one-step application [16,19]. Considering these features, dissolving MNs have been

successfully proposed to optimise the delivery of nanoparticles (NPs) into the skin [20,21]. The combination of MNs and NPs technologies solves several issues, including drug solubility, stability, permeability, half-life and targeted drug delivery [22]. Notably, recent works have revealed the benefits of including liposome-like nanovesicles in dissolving MNs to sustain a wide range of biomedical applications, including cancer [23–25], dementia [26,27], vaccination [28], osteoarthritis [29], and psoriasis [30].

Based on the previous considerations, herein, we present an RB delivery platform consisting of RBTF loaded into a trilayer dissolving MN array (TDMNs). The use of a trilayer MN array instead of a standard one derives from the need to contrast the supposed softening effect exerted by lipid and surfactant materials; furthermore, due to the photo-instability of RB, the third layer has been added to prevent undesired drug degradation. The study aims to provide a suitable pharmaceutical form for delivering RBTF intradermally as an adjuvant tool in early-stage melanoma surgical management. In this view, the RBTF-loaded TDMNs (RBTF-TDMNs) were developed to be applied after the surgery to eradicate cancer cells that escaped the medical procedure [31]. Following this approach, only the effective melanoma lesion would be removed, reducing the known surgical margins of 5 cm and consequently the patient's recovery without posing a risk for a late recurrence. Mainly, TDMNs were selected to provide a controlled dosage and release of RBTF beyond the SC without modifying the essential RBTF physicochemical features. Experimentally, a previous preformulation study adapted the already developed RBTF for integrating them into the polymeric matrix; then, the optimised RBTF were used to prepare the RBTF-loaded TDMNs (RBTF-TDMNs). The final array was evaluated in terms of physicochemical and mechanical properties, both in-vitro and ex-vivo. The physical stability of RBTF following the integration in RBTF-TDMNs was also assessed. Finally, a dermatokinetic study was performed using Franz diffusion cells to determine the intradermal delivery efficiency of the novel RBTF-TDMNs array.

2. Materials and Methods

2.1 Materials

Rose Bengal disodium salt (RB), cholesterol, SpanTM 80, HPLC grade methanol, ammonium acetate, sodium hydroxide, phosphate-buffered saline (PBS) tablets (pH 7.3–7.5), poly (vinyl alcohol) (PVA) (9–10 kDa) and dimethyl sulfoxide (DMSO) were purchased by Sigma-Aldrich (St. Louis, MO). Polyvinylpyrrolidone (PVP) PlasdneTM K-29/32 (58 kDa) and PlasdneTM K-90 (1,570 kDa) were

obtained from Ashland (Wilmington, Delaware, U.S). Lipoid S 100 was obtained by Lipoid GmbH (Ludwigshafen, Germany). Uranyl acetate (gadolinium acetate tetrahydrate) was purchased by Ted Pella Inc (Redding, CA). Ethanol (industrial methylated spirit 99.5%) was obtained by Atomic Scientific (Manchester, UK). Ultrapure water was obtained from a water purification system (Elga PURELAB DV 25, Veolia Water Systems, Dublin, Ireland). Silicone elastomer (Silastic™ RTV-4250-S BASE) and curing agent (Silastic™ RTV-4250-S Silicone Curing Agent) were purchased from Notcutt Ltd, Surrey, UK. Reagent A (LSR9-9508–30 Part A Silicone Elastomer) and Reagent B (LSR9-9508–30 Part B Silicone Elastomer) were purchased from Polymer Systems Technology Ltd.

2.2 Preparation and characterisation of RB-loaded TF

2.2.1 Preparation of RB-loaded TF

RB-loaded TF (RBTF) were prepared by a reverse-phase evaporation method (REV) as already described by the same authors [14], with slight modifications. The lipid mixture, consisting of 142 mg of Lipoid S 100, 26 mg of cholesterol and 14 μ L of Span™ 80, was dissolved in 5 mL of ethanol and homogenised with 10 mL of RB aqueous solution (RB=2 mg/mL) by a probe-type ultrasonic device (Davidson & Hardy Ltd cooperating with Fisher Scientific, Leicestershire, UK) (60 s, 50% ultrasound (US) amplitude). Following this, ethanol was removed by rotary evaporation (Rotavapor, Buchi Labortechnik, Switzerland) under vacuum at 50°C, and the resulting dispersion was kept for 1 h at room temperature. Finally, RBTF dispersion was further submitted to six probe sonication cycles, each consisting of 10 s of 50% US amplitude followed by an interval of 20 s [32].

2.2.2 Particle Size and Zeta Potential analysis

The particle size, polydispersity index (PDI) and zeta potential were analysed using a NanoBrook Omni particle sizer and zeta potential analyser (Brookhaven, New York, USA). Two batches of RBTF dispersion were analysed in triplicate (n=6). Dynamic light scattering (DLS) was employed to measure the hydrodynamic size and the PDI. Before each measurement, samples were diluted with ultrapure water to ensure that the concentration was within the required range of the instrument. The analyses were performed using the following conditions: fluid refractive index 1.333; temperature 25 °C; viscosity 0.890 cP; scattering angle 90°; equilibration time 3.0 minutes; and sample run time 300 s. Zeta potential was measured through the Phase Analysis Light Scattering (PALS) technique after diluting samples with ultrapure water. The analyses were performed at 25°C, the equilibration time was 3.0 minutes, and the sample run time was 300 s.

2.2.3 Morphological characterisation

RBTF morphology was investigated by microscopical techniques, including transmission electron microscopy (TEM) and multi-photon microscopy. For TEM analysis, one drop of the sample was added on top of a carbon copper grid and stained with uranyl acetate. After 20 minutes, samples were washed with deionised water and dried. Following this, the grids were placed on the specimen holder. Images were acquired using a Jeol JEM-1400 Transmission electron microscope (Welwyn Garden City, UK) at the acceleration voltage of 120,000 V and magnification between x1000 and x50,000. Images were visualised with a Jeol Ruby 8MB bottom-mounted CCD camera. Fluorescence images were collected using Leica TCS SP8 multi-photon scanning microscope (Leica Microsystems (UK) Ltd, Milton Keynes, UK) equipped with an upright DM6 microscope body and a motorised stage. Leica Application Suite X software (3.5.7.23225) was used for image acquisitions. Samples were excited with 549 nm laser lines from the Mai Tai Deep See Mode-Locked laser system (Newport-Spectra Physics, UK). Fluorescence emission was collected via HyD GaAsP-spectral detectors for Rose Bengal dye between 557nm-660nm. A water immersion objective HC FLUOTAR L 25x with 0.95 Numerical Aperture or dry objectives HC PL APO 10X/0.40 NA or HC PL FLUOTAR L 40X0.60 NA were used for image acquisition as appropriate. Fluorescence images were collected at 1024x1024 pixel resolution format and a scanner speed of 400 Hz. Image analysis was performed using the Leica Application Suite X software (3.7.020979).

2.2.4 Evaluation of RB content in RBTF dispersion

To determine RB content in RBTF dispersion, RBTF vesicles were disrupted by dissolving the dispersion in methanol with the help of a thermomixer (Eppendorf ThermoMixer™ F1.5, Hamburg, Germany) set at 56° C for 20 minutes at 1,5000 rpm. RB amount was then quantified by UV-Vis spectrophotometry (BMG LABTECH Ltd, Bucks, UK) and calculated using the calibration curve prepared in methanol (standard solution in the range of 1–12 µg mL⁻¹; $y = 0.092x + 0.0159$; $R^2 = 0.9997$). Finally, the drug content (DC%) was determined by the following equation:

$$DC\% = \frac{RB \text{ quantified in RBTF dispersion}}{\text{Theoretical amount of RB used}} \times 100 \quad (1)$$

Three batches of RBTF were analysed in triplicate (n=9).

2.3 Fabrication and morphology of RBTF-loaded TDMNs

A mould-casting technique was employed to obtain the RBTF-loaded TDMNs (RBTF-TDMNs) (Li et al., 2022b; Paredes et al., 2021). The fabrication process is illustrated in Fig. 1.

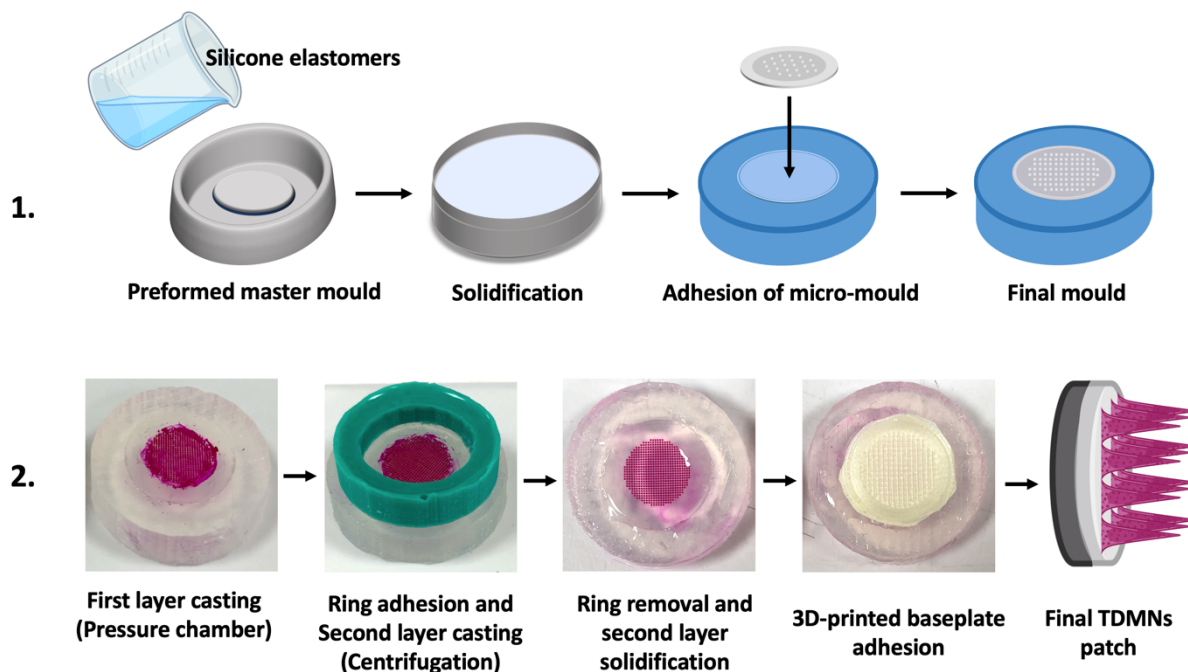


Fig. 1. Schematic illustration of the fabrication process of RB-TDMNs and RBTF-TDMNs. 1.1 Fabrication of the mould. 1.2 Preparation of TDMNs.

The primary mould was obtained by pouring silicone elastomers into a preformed master mould and left to solidify overnight at room temperature. The following day, a micro-mould (600 pyramidal needles of 750 μm height on a 0.76 cm^2 area) was stuck on the primary mould using silicone elastomers and dried at 80°C for 20 min, obtaining the final mould [33]. A previous preformulation study identified the most suitable composition of the first layer, which is the one loading the TF (supplementary material). To prepare the first layer, the RBTF dispersion was concentrated up to 1 mL removing the water by rotary evaporation at 50° C under vacuum. Then, the RBTF dispersion was mixed with an equal portion of an aqueous polymer blend [27] made of 40% w/w of PVA and Pladone™ K-29/32 (1:1 ratio) in a DAC 150 FVZ SpeedMixer™ (High Wycombe, England) (3,500 rpm, 3 min). The resulting formulation was poured in excess on the top of the final mould and pressed into the needle cavities using a positive pressure chamber (Protima®, TUV Rheinland, Aliquippa, PA, US) (5 bar, 4 minutes). Then, the excess of the formulation was carefully removed with a spatula, and the mould was placed in the pressure chamber (5 bar) for 30 minutes; the first layer was finally allowed to dry at room temperature. Afterwards, a ring insert was attached to the silicone mould using PVA 40% w/w as glue, and the second layer (RBTF-free), consisting of 300 μL of Pladone™ K-90 30% w/w in water, was cast and centrifuged at 5,000 rpm for 4 minutes followed

by the immediate removal of the holder ring. After drying at room temperature, the third layer, the baseplate (BP), was applied. The BP was round, with a diameter of 13 mm and a thickness of 0.6 mm; it was 3D-printed using an Ultimaker 3 (Ultimaker B.V., Geldermalsen, The Netherlands) with a 0.4 mm extruder nozzle loaded with PLA filament. The BP was first attached to 3M Microfoam™ tape and then adhered to the needle base using double-sided tape. Finally, RBTF-TDMNs were carefully peeled off from the mould. Free drug-loaded TDMNs (RB-TDMNs) were prepared following the same procedure but using 1 mL of RB aqueous solution (RB=20 mg) instead of the RBTF dispersion to prepare the first layer. To evaluate the morphological properties, RBTF-TDMNs and RB-TDMNs were visually examined by optical microscopy using a Leica EZ4W stereomicroscope (Leica Microsystems, Milton Keynes, UK), and the multi-photon microscopy as previously described in section 2.2.4.

2.4 Determination of RB content in RB-TDMNs and RBTF-TDMNs

RB amount localised to the needle area of RB-TDMNs and RBTF-TDMNs was evaluated. Briefly, BP was first detached from the TDMNs. The array area containing the MNs, including the second layer where the needle tips were held, was carefully isolated using scissors, added to 5 mL of methanol, and magnetically stirred until the complete disruption (about 30 minutes). Then, 1 mL was taken and centrifuged for 15 min at 14,000 rpm (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) to precipitate the polymer. The supernatant was separated and further mixed with the help of a thermomixer set at 56°C for 20 min to ensure the complete RB extraction. Finally, RB analysis was carried out by HPLC coupled with a fluorescence detector (FLD) (Agilent Technologies 1220 Infinity UK Ltd, Stockport, UK). The chromatographic column employed was a C18 Phenomenex SphereClone™ column ODS (1) (150 x 4.6 mm i.d. with 5 µm particle size) (Phenomenex Cheshire, UK). The mobile phase was a mixture of methanol and ammonium acetate buffer pH 8 (60:40 v/v), with a 1 mL/min flow rate. The excitation and emission wavelength were set at 556 nm and 573 nm, respectively, the injection volume was 40 µL, and the run time was 6 min. Four RB-TDMNs and RBTF-TDMNs were separately analysed (n=4).

2.5 Physical stability of RBTF in RBTF-TDMNs

The physical stability of RBTF in RBTF-TDMNs was determined by analysing the main characteristics of the nanovesicles following the integration process in the RBTF-TDMNs. The RBTF-TDMNs were added to 5 mL of ultrapure water to dissolve the polymer and redisperse RBTF

completely. The redispersed RBTF (red-RBTF) were evaluated in size, PDI, zeta potential value, and morphology, as described in subsections 2.2.2 and 2.2.3. Results of red-RBTF were compared to the original RBTF. Three RBTF-TDMNs were separately analysed three times (n=9).

2.6 In-vitro and ex-vivo mechanical properties testing of RB-TDMNs and RBTF-TDMNs

Mechanical strength and insertion capability were evaluated using a TA.XT2 Texture Analyser (Stable Micro Systems., Ltd., Haslemere, UK) as illustrated in Fig. 2.

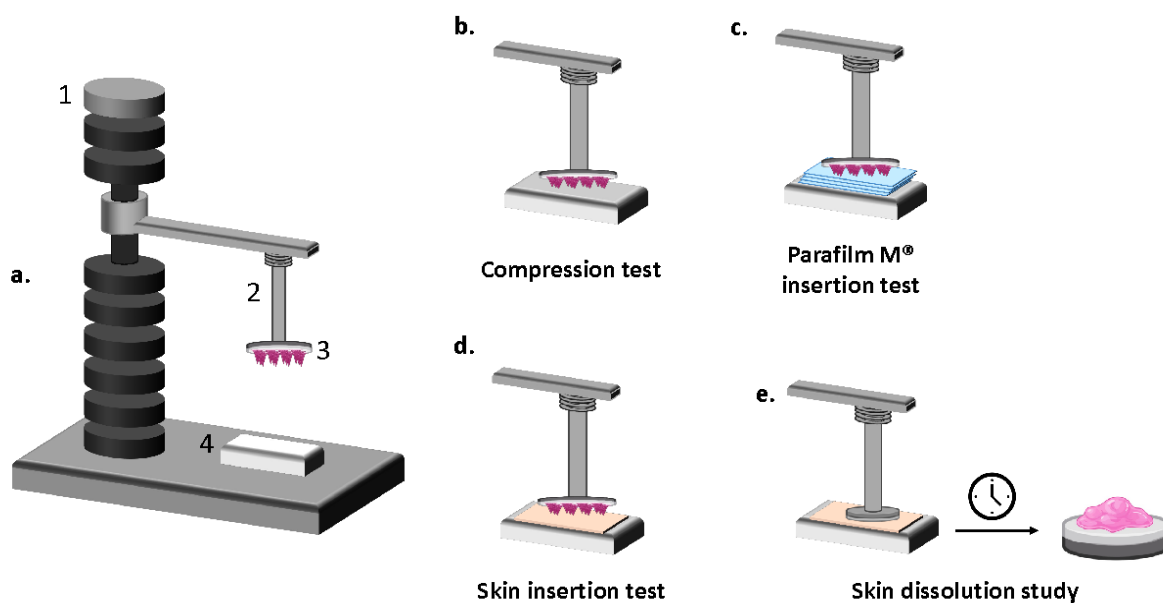


Fig. 2. Mechanical properties testing of TDMNs. a) Diagram of TA.XT2 Texture Analyser. 1. Texture analyser; 2. Movable aluminium probe; 3. TDMNs patch; 4. aluminium block. b) Testing of TDMNs' ability to withstand compression. c) Insertion in Parafilm M™ skin simulant model. d) Insertion capability in full-thickness porcine skin. e) Study of the TDMNs dissolution time in full-thickness porcine skin.

The TDMNs were attached to the probe of the texture analyser, and the MNs pressed vertically against a flat aluminium surface, applying a force of 32 N for 30 s at a speed of 1.19 mm/s. These conditions simulate the average human force applied during MN array administration [34]. The ability of TDMNs to withstand the compression was expressed in terms of percentage height reduction (HR%), calculated according to the equation:

$$HR\% = \frac{H_a - H_b}{H_a} \times 100 \quad (2)$$

Where H_a is the height of the MNs before the compression test, and H_b is the height of the MNs after the compression test. Three RBTF-TDMNs and three RB-TDMNs were separately evaluated; precisely, 24 MNs of each TDMNs were analysed ($n=72$).

Insertion capability was evaluated using the same experimental set-up. The in-vitro insertion study was performed by pressing the MNs against a skin simulant Parafilm MTM model described by Larrañeta and colleagues [34] and widely used in the literature. The Parafilm MTM (Bemis Company Inc., Soigneis, Belgium) was cut to form eight layers stacked one on top of the other with a thickness of 1 mm (approximately 0.125 mm per layer) (Larrañeta et al., 2014; Li et al., 2022b). The insertion was expressed in the number of perforations in each Parafilm MTM layer, determined under a Leica EZ4W stereomicroscope using two polarised filters [17]. Three RBTF-TDMNs and three RB-TDMNs were separately evaluated ($n=3$). The insertion capability of the TDMNs was tested ex-vivo using the full-thickness neonatal porcine skin. First, the skin was obtained from stillborn piglets, kindly donated by the Agri-Food and Bioscience Institute (Hillsborough, Northern Ireland, UK); piglets were immediately frozen after birth at -20°C and defrosted overnight before use. Full-thickness skin was excised using a surgical scalpel and carefully shaved using a disposable razor (Gillette Blue IITM, Gillette, Reading, UK). The skin was allowed to equilibrate for 30 minutes in PBS pH 7.4 before the experiment. The Texture Analyser was employed to perform the experiment, using the same set-up above described. The insertion depth was evaluated by multi-photon microscopy, as described in section 2.2.4.

2.7 Ex-vivo skin dissolution study

To evaluate the dissolution time, RB-TDMNs and RBTF-TDMNs were pressed against the skin, previously prepared as reported in section 2.6, and then removed at different time points (1 min, 3 min, 5 min, 10 min) (Fig. 2). The TDMNs were morphologically evaluated at each time point using a Leica EZ4 D stereo microscope.

2.8 Ex-vivo dermatokinetic study

The dermatokinetic profile of RB-TDMNs, RBTF-TDMNs and RBTF dispersion was evaluated ex-vivo across full-thickness neonatal porcine skin. The skin was obtained and prepared as previously described in section 2.6. The experiment was performed using Franz diffusion cells (Permeagear, Hellertown, PA, USA) with a 1.77 cm² orifice area and 12 mL receptor volume. The receiver compartment was filled with 12 mL of degassed PBS pH 7.4 as the receiver medium. Meanwhile, the

skin was fixed on the donor compartment with the help of ethyl 2-cyanoacrylate glue (Gorilla Glue Europe Ltd, Chorley, UK), with the SC side facing the donor compartment. RB-TDMNs and RBTF-TDMNs were separately inserted into the full-thickness neonatal porcine skin using the manual force of the same operator for 30 s. Then, the donor chamber was carefully allocated on the top of the receiver compartment, with the inner side facing PBS. A cylindrical stainless-steel weight (5 g) was placed upon the inserted TDMNs to avoid movement or ejection. In the case of RBTF dispersion, the formulation was added onto the skin surface within the donor compartment (0.1 mL, 100 µg of RB), already mounted on the top of the receiver compartment. The system's temperature was set at $37\pm1^{\circ}\text{C}$, and an equilibration time of 30 min was allowed before starting the experiment. The donor and the sampling ports were sealed using Parafilm MTM to minimise evaporation and contamination; the magnetic stirrers were set at 600 rpm to homogenise the receiver medium. The skin samples collected at each predetermined time point (1h, 3h, 6h, 12h, 24h) were carefully cleaned and placed on a hot plate for 2-3 min at 60°C to separate the epidermis from the dermis. To extract RB from the skin samples, 0.5 mL of distilled water was added to the samples and disrupted at 50 Hz for 15 min by a Qiagen TissueLyserTM LT (UK Quiagen Ltd, Manchester, UK). Subsequently, 1 mL of DMSO was added, and the samples were further homogenised for 15 min (50 Hz); this step was repeated twice to ensure the total RB extraction from the skin. Following a centrifugation process (14,000 rpm, 15 min), the amount of RB in the supernatant was determined using HPLC (section 2.4). Three RBTF-TDMNs, RB-TDMNs and RBTF dispersion, were separately analysed (n=3).

2.9 Statistical analysis

All data in this study were presented as means $\pm$ standard deviation (SD) of the mean and were calculated using GraphPad PrismTM version 9.3.1 (GraphPad Software, San Diego, California, USA). The same software was used to determine statistical differences by Student's t-test and analysis of variance (ANOVA). A value of $p < 0.05$ was noted as statistically significant.

3. Results and Discussion

3.1 Preparation and characterisation of RBTF

Before the integration of RB in the TDMNs, RB was formulated in TF by a reverse-phase evaporation technique, as described in our previous work [14]. A preformulation study, detailed in the supplementary material, was performed on blank TF to evaluate if the original formulation was

suitable for the integration into the polymeric matrix of PVA and PVP. Results revealed that the initial lipid concentration (Lipoid S100 and cholesterol, 47.5 mg/mL) was excessive, hampering the proper MNs formation. Therefore, we decreased the lipid concentration to 16.88 mg/mL, leading to MNs with promising morphological (Fig. S1, supplementary material) and mechanical properties (Fig. S2, supplementary material). Herein denoted as RBTF, the new formulation was developed, maintaining the ratio between Lipoid S100, Span 80 and cholesterol unchanged compared to the original formulation.

The size of RBTF measured 62.91 ± 6.28 nm with a PDI of 0.271 ± 0.045 , and the zeta potential was -38.47 ± 0.20 mV. DLS analyses indicated a dispersion in the nano-sized range, suitable to be included in dissolving MNs [24,27,29]. The PDI value was lower than 0.3 and the zeta potential higher than 30 mV, predicting the thermodynamic stability of the formulation [20,35]. Herein, no dimensional stability test overtime of RBTF has been performed; nevertheless, the PDI and zeta potential results were in line with those presented in our initial work [14], where the formulations proved to be physically stable over 60 days. Furthermore, NPs instability is characteristic of NPs dispersed in a liquid vehicle, where aggregation is more likely to occur. This phenomenon can be easily prevented by solvent removal and conversion into a solid-like dosage form, such as TDMNs [20,36,37].

TEM micrographs (Fig. 3.a, b, c) showed spherical-shaped particles. The analyses confirmed the proper formation of RBTF since the phospholipid bilayer, the primary indication of a successful liposome-like structure, is clear and easily identifiable. Considering the size lower than 100 nm and TEM evaluations, RBTF are defined as small unilamellar vesicles (SUV) [38]. However, it is essential to note that DLS reports an overall average diameter of the dispersed RBTF. In conjunction with the PDI value and TEM micrographs, the presence of vesicles larger and smaller than 100 nm is highly probable.

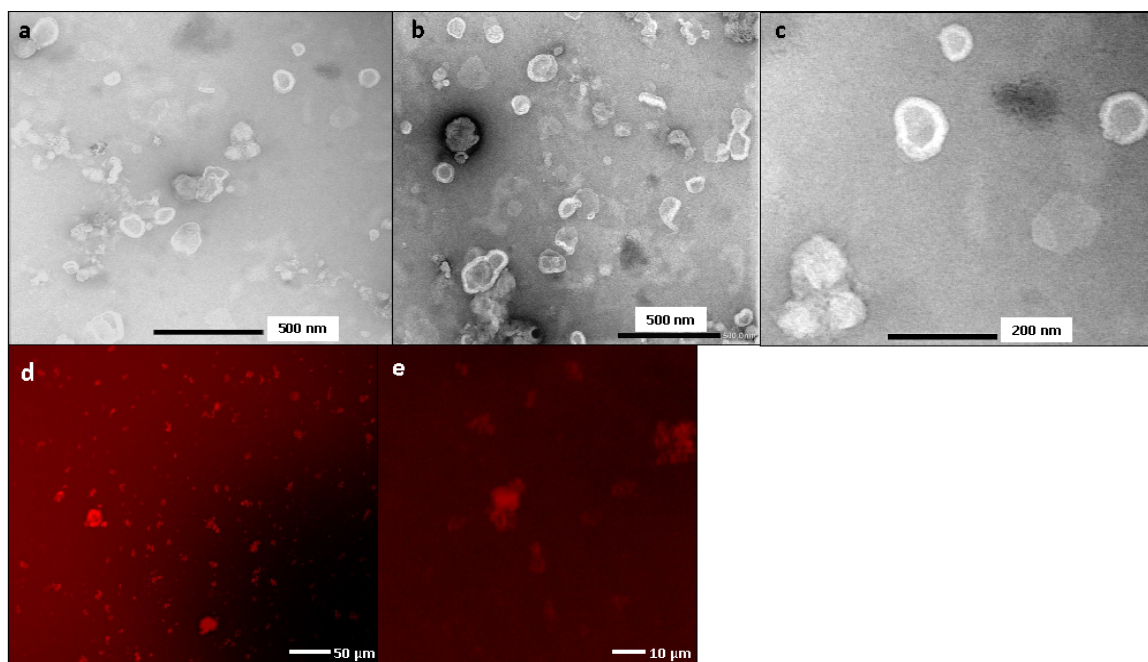


Fig. 3. Morphological evaluation of RBTF. a, b). TEM micrographs, 500 nm scale bar, magnification x20K; c. TEM micrograph, 200 nm scale bar, magnification x80K. d, e). Multi-photon micrographs, scale bar 50 μm and 10 μm, respectively.

Finally, the RB content in RBTF was $110.31 \pm 3.49\%$. It is important to cite that the term “RBTF” refers to RB-loaded nanovesicles dispersed in the water phase; hence, the RB content is intended in the whole dispersion made of the nanovesicles and their dispersing vehicle. Herein, no physical isolation of nanovesicles was performed. This assay evaluated the chemical stability of the drug through the manufacturing process and revealed that no degradation phenomena occurred. RB has a high affinity for lipids and undoubtedly interacted with the lipid phase of the RBTF, as also proved by the negative zeta potential value [14,39–41].

3.2 Fabrication and morphological characterisation of RB-TDMNs and RBTF-TDMNs

In this study, a trilayer dissolving MN array was proposed to sustain the intradermal delivery of RBTF. The morphology of RB-TDMNs and RBTF-TDMNs was first investigated through optical microscopy (figure 4, a-e). To better characterise the new RB delivery platform, a 3D reconstruction of the TDMNs via multi-photon fluorescence microscopy has been carried out; herein, the view from different perspectives is proposed (Fig. 4, f-i).

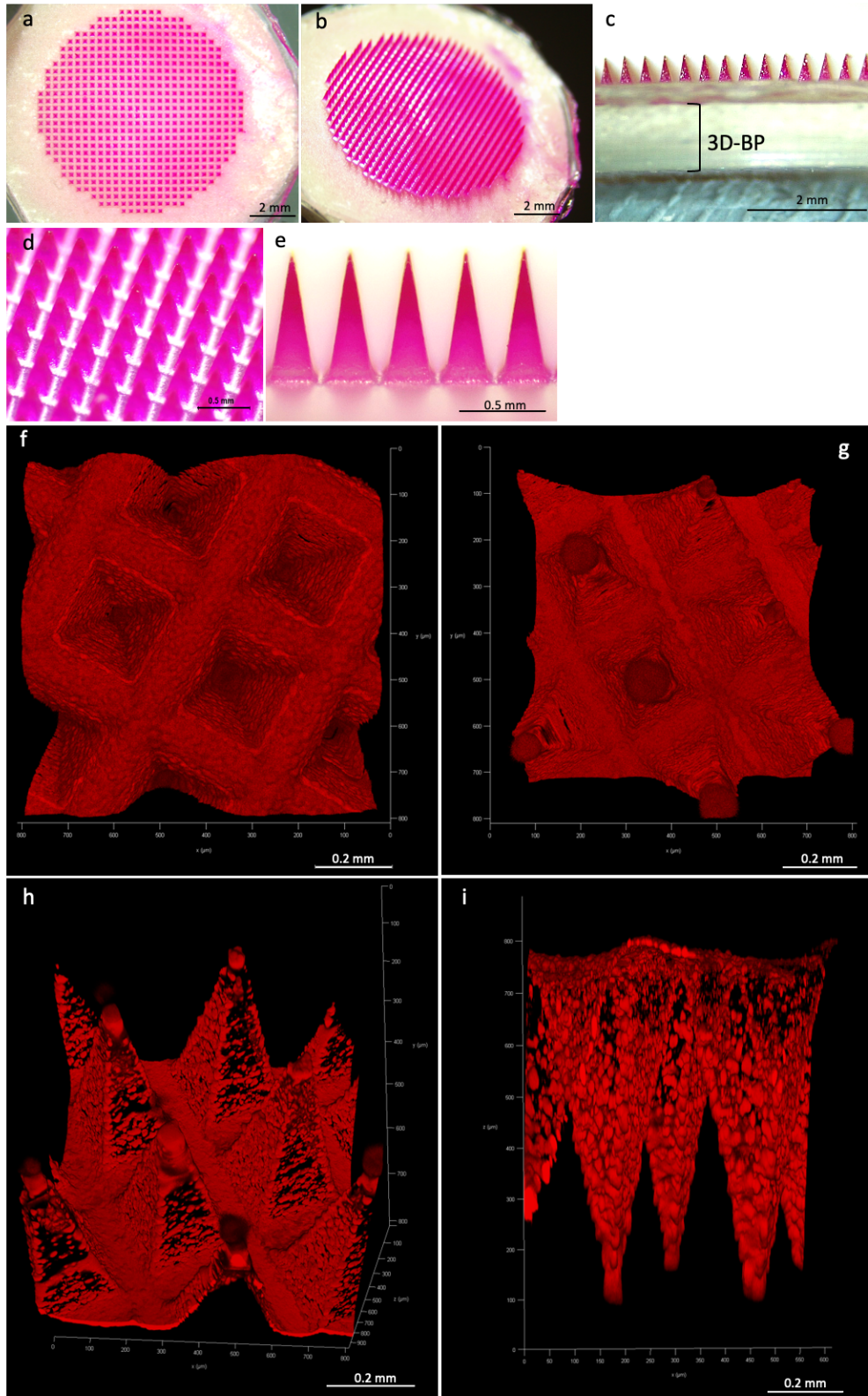


Fig.4 Morphology of TDMNs. a-b) top view of TDMNs by optical microscopy. Scale bar 2 mm, magnification 8x. c) Side view, focus on trilayer TDMNs structure by optical microscopy. Scale bar 2 mm, magnification 8x. d) top view of TDMNs by optical microscopy. Scale bar 0.5 mm, magnification 35x. e) tips of TDMNs. Scale bar 0.5 mm, magnification 35x. F-i) imaging of TDMNs obtained by the 3D reconstruction by multi-photon microscopy. Scale bar 0.2 mm.

RBTF-TDMNs and RB-TDMNs were visually identical; the morphological characterisation demonstrated the successful formation of the 600 micro projections pyramidal-shaped, bubble-free, sharp, and uniformly distributed on the patch area. To reduce the drug waste, the pharmaceutical cargo was loaded only in the first layer of the TDMNs, formulated using an aqueous blend of low molecular weight PVA and PVP. It is known in the literature that the combination of these polymers forms tips with better mechanical performance than a single polymer due to the formation of hydrogen bonds amongst C=O groups of PVP and -OH groups of PVA [22]. Accordingly, in the preformulation study reported in the supplementary material, the combined PVA/PVP showed the best penetrability (Fig. S2, supplementary material). The first layer's formulation was forced to fill the cavities of the micro-mould by applying positive pressure, followed by water evaporation and the application of the viscous second layer by centrifugation in the presence of a ring insert. This method allowed the formation of the entire MNs and, in the meantime, assured an excellent adhesion between the two layers [17]. Finally, as the third layer, the PLA 3D-BP was attached. The 3D-BP is expected to maximise the system's overall efficiency, thus minimising the supposed softening effect caused by the semi-solid Lipoid S100 and Span™ 80; furthermore, PLA was reported to provide added mechanical strength to the MNs [22], the pyramidal geometry of the tips was selected for the same reason (Li et al., 2022b; Salwa et al., 2021). Moreover, considering that the cutaneous route is exposed to light, the use of a non-transparent 3D-BP acts as a barrier protecting RB from photodegradation.

3.3 Evaluation of RB content in RB-TDMNs and RBTF-TDMNs

Results showed that $139.31 \pm 22.16 \mu\text{g}$ were loaded in RB-TDMNs, and $64.37 \pm 8.61 \mu\text{g}$ in RBTF-TDMNs (p value < 0.001). No degradation processes occurred during the preparation of RBTF (section 3.1), and the two TDMNs were prepared following the same procedure. The lower RB amount in RBTF-TDMNs compared to RB-TDMNs can be attributed to a higher viscosity of the final polymer blend incorporating the mix of lipid and surfactant constituting RBTF (first layer), which may result in a greater resistance in filling the micro-mould, which is responsible for the drug loading. This phenomenon has already been observed in the literature and is more common in the case of drugs with a physical-chemical profile similar to RB [42]. The RB amount localised to the MNs area was calculated once the system was entirely dried; the needle tips were not separated from the thin second layer attached. Indeed, given an intradermal delivery, the residual amount of RB eventually migrating from the tips to the second layer is likely to deposit into the skin through the

microchannel created by the insertion of the array and exploiting the permeation properties of the TF technology. The RB amounts mentioned above determined the drug dose of RB-TDMNs and RBTF-TDMNs, respectively, and were used for further evaluations.

3.4 Physical stability of RBTF in RBTF-TDMNs

RBTF were integrated into TDMNs to provide a final pharmaceutical form to control the dosage, circumvent eventual stability issues and ensure the RBTF delivery to the target site. With these premises, it is essential to define the physical stability of RBTF in RBTF-TDMNs. Hence, RBTF-TDMNs were dissolved in water and then redispersed RBTF (Red-RBTF) were evaluated. Red-RBTF measured 183.62 ± 29.26 nm with a PDI of 0.224 ± 0.064 and a zeta potential value of -38.19 ± 0.14 mV. As already observed in similar investigations [24,29], particle size significantly increased compared to the original RBTF (p value < 0.001), whereas PDI and zeta potential values did not statistically differ. TEM investigation proved that red-RBTF were spherical and unilamellar (Fig. 5. a-c); no appreciable differences were seen under the multi-photon microscopy (Fig. 5. d).

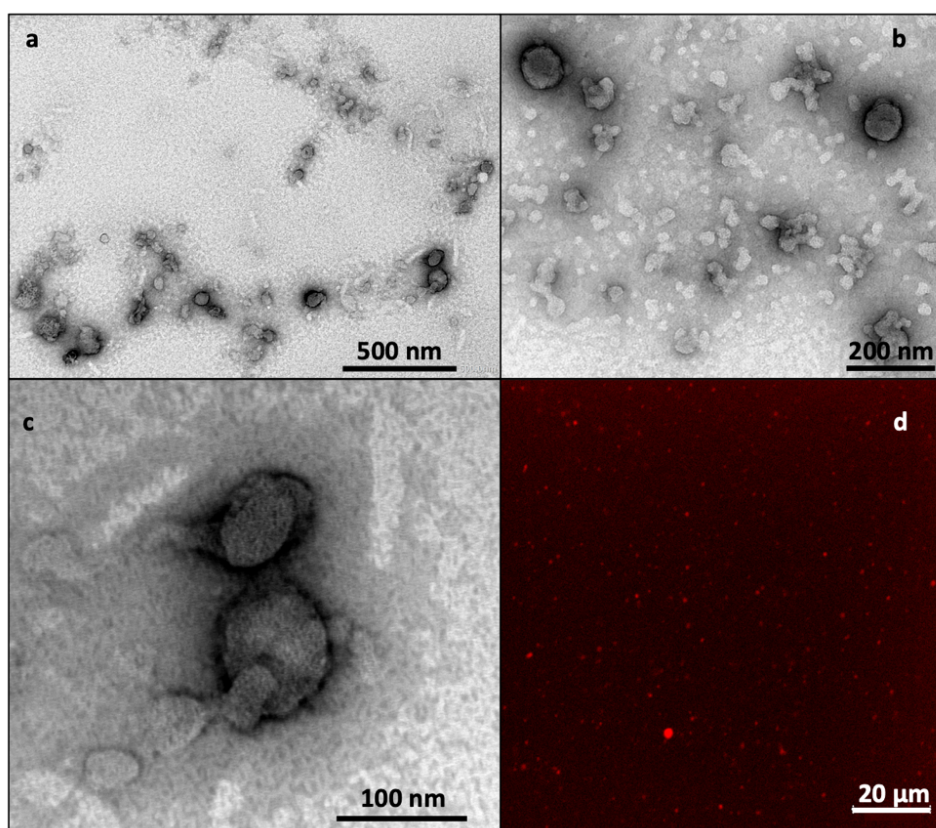


Fig 5. Microscopical evaluation of red. RBTF. a. TEM micrographs, 500 nm scale bar, magnification x20K; b. TEM micrograph, 200 nm scale bar, magnification x80K; c. TEM micrograph, 100 nm scale bar, magnification x80K. d. multi-photon micrographs, scale bar 20 μ m.

As previously discussed (section 3.1), DLS provides an overall average diameter of the NPs, which can be affected by several factors [37]. As demonstrated in our previous work [14], RB is located inside the RBTF and intercalated beneath the phospholipid bilayer. Considering the log P of RB (0.59), we cannot exclude interactions between the polymers used and the surface of the RBTF. Indeed, the interaction between RB and PVA has been already described in the literature [43], and non-well-defined structures beyond Red-RBTF were seen in the TEM micrographs, which can be hypothetically referred to as polymer residues. However, the particle size recorded is in line with the one observed in our previous work, which efficiently promoted RB permeation through the epidermis [14]. The zeta potential was unchanged, indicating that RB was still efficiently interacting with the lipid vesicles [14,41]. TEM evaluations showed that Red-RBTF maintained the same structure as the RBTF before the integration process, which is the essential feature for the drug encapsulation and the intact RBTF delivery to the deepest skin strata [24].

3.5 In-vitro and ex-vivo mechanical properties of TDMNs

The effective penetration of the MNs into the skin is imperative to achieve the desired delivery goals. Therefore, we evaluated RBTF-TDMNs and RB-TDMNs' penetration ability and their mechanical resistance to the insertion force required. Human skin exhibits unique characteristics that vary depending on gender, race, proper physiological conditions, or other factors [44]. Consequently, the mechanical force necessary to assure an effective MNs insertion is not standard. However, a model method was proposed to provide a standard reference for testing MNs insertion and was adopted here as described in section 2.6 [34]. Fig. 6 reports the results of the in-vitro mechanical testing.

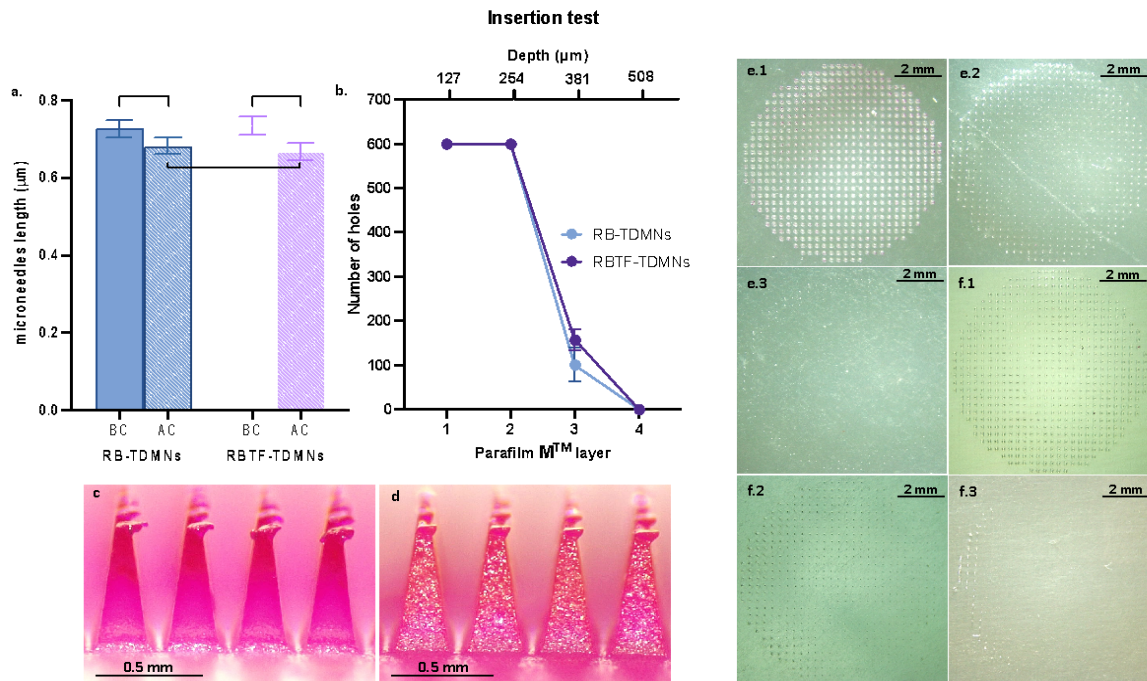


Fig. 6. *In vitro* mechanical testing of TDMNs. a) MN height before and after the compression test ($n=3$). **** p value <0.0001 ; ** p value <0.01 . b) Insertion capability in Parafilm M™ skin simulant model ($n=3$). c) MNs of RBTF-TDMNs acquired with the optical microscope after the compression test. Scale bar 0.5 mm, magnification 35x d.) MNs of RB-TDMNs after the compression test acquired with the optical microscope. Scale bar 0.5 mm, magnification 35x e) Parafilm M™ sheets following the insertion of RBTF-TDMNs: first layer (e.1), the second layer (e.2), the third layer (e.3). f) Parafilm M™ sheets following the insertion of RB-TDMNs: first layer (f.1), second layer (f.2), and third layer (f.3).

RB-TDMNs reported an MNs height reduction of $6.53 \pm 0.51\%$ following the 32 N compression force application against a flat solid aluminium surface. In contrast, RBTF-TDMNs suffered from a statistical higher reduction, reaching a value of $9.77 \pm 0.18\%$ (p value <0.0001) (Fig. 6.a). The images acquired by optical microscopy (Fig. 6.c, d) showed that the MNs were sharp and pyramidal-shaped before the test. The height reduction was visible following the compression, and the tips appeared pressed but not crushed or bent. The insertion test in the Parafilm M™ skin simulant model proved that RBTF-TDMNs and RB-TDMNs entirely pierced the first two layers (Fig. 6. b, e, f), corresponding to a depth of $\sim 0.254 \mu\text{m}$. The insertion decreased in the third layer ($\sim 0.381 \mu\text{m}$), creating 100-157 holes without significant differences between the two TDMNs. Even if the incorporation of lipid materials and surfactant decreased the resistance to compression, this did not appear to affect the insertion ability. Indeed, previous studies reported that an MN height reduction not above 10% and the insertion of at least two Parafilm M™ layers are a prerequisite for an efficient penetration ability, and these parameters were here respected [17,45,46].

To better predict the in-vivo performance of RBTF-TDMNs and RB-TDMNs, we further evaluated their penetration ability ex-vivo in full-thickness porcine skin following the same method. In this

case, the insertion was tracked under multi-photon microscopy by exploiting the fluorescence properties of RB [47]. Multiphoton fluorescence microscopy has become a very effective tool for studying skin. It provides high-resolution 3D visualisation of the analysed tissue to evaluate the accumulation and penetration properties of drugs released from cutaneous administration [48,49]. The TDMNs insertion in the skin is illustrated in Fig.7. Briefly, Fig.7a provide an overview of the test: Fig 7.a1 shows only a TDMNs (red-coloured) penetrating the skin (grey-coloured) isolated by the instrument software, Fig 7.a2 represents only the skin (grey-coloured) penetrated by the TDMNs isolated by the instrument software, and Fig 7.a3 shows both the TDMNs and the skin. Fig 7. b-c depicts only the TDMNs penetrating the skin, processed as Fig 7.a3. Fig 7.d shows the skin following the physical detachment of the RBTF-TDMNs from the skin itself; thus, it shows the residues of TDMNs remaining in the skin once they have been removed.

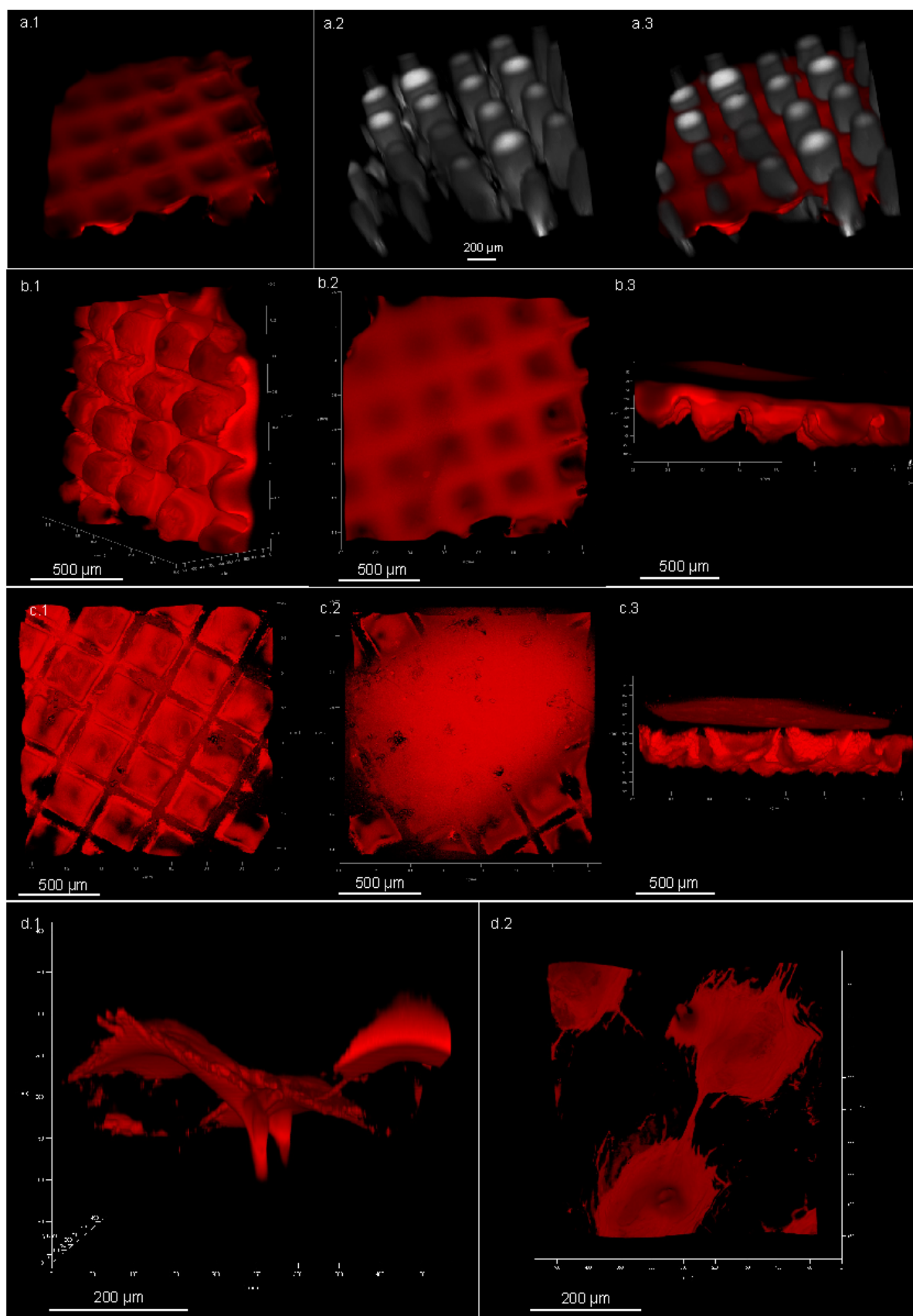


Fig. 7. 3D visualisation of the *ex vivo* TDMNs insertion in full-thickness skin by multi-photon fluorescence microscopy. a) Representation of TDMNs inserted in the skin; a.1) TDMNs inserting the skin isolated by the instrument's software; a.2) skin isolated by the instrument's software. a.3) full image of TDMNs inserted skin. Scale bar 200 μm . b) RB-TDMNs inserted in the skin, scale bar 500 μm ; b.1) bottom-side view; b.2) top view;

b.3) lateral view. c) RBTF-TDMNs inserted in the skin, scale bar 500 μm ; b.1) bottom view; b.2) top view; b.3) lateral view. d) skin after the removal of RBTF-TDMNs, scale bar 200 μm . d.1) lateral view; d.2) bottom view.

During the analyses, liposome-like structures were detected on the surface of RBTF-TDMNs when RBTF-TDMNs penetrated the skin (Fig. 7.c) and were absent in the case of RB-TDMNs (Fig. 7.b). Beyond this, the multi-photon fluorescence technique confirmed the penetration ability of both the TDMNs previously anticipated by the in-vitro Parafilm MTM insertion test. Indeed, the results from the ex-vivo insertion study (Fig. 7.b-c) indicated that the TDMNs could penetrate to a depth range of approximately 400-450 μm , similar to the 375 μm observed in the Parafilm MTM; furthermore, the MNs structure is still recognisable into the skin following the removal of the TDMNs from the skin itself (Fig. 7.d). It was observed that the TDMNs penetration depth, both in-vitro and ex-vivo, was considerably lower than the TDMNs length, which ranges around 750 μm . The phenomenon is explained by the inherent viscoelastic properties of both Parafilm MTM and skin, which resist MNs penetration, resulting in an incomplete insertion [42]. However, the thickness of the entire epidermis is approximately $83.7 \pm 16.6 \mu\text{m}$, of which $14.8 \pm 4.8 \mu\text{m}$ are represented by the SC and the remaining by the viable epidermis [50]. The presented data proved that RB-TDMNs and RBTF-TDMNs could fully penetrate the epidermis, releasing the active cargo up to the dermis, de facto potentially covering the area affected by early-stage melanoma.

3.6 Ex-vivo skin dissolution study

Considering that the effective active cargo release depends on the dissolving ability of the polymeric tips, the TDMNs dissolution study following application to the skin was performed to determine the time required for complete MNs dissolution (Fig. 8) [29,46].

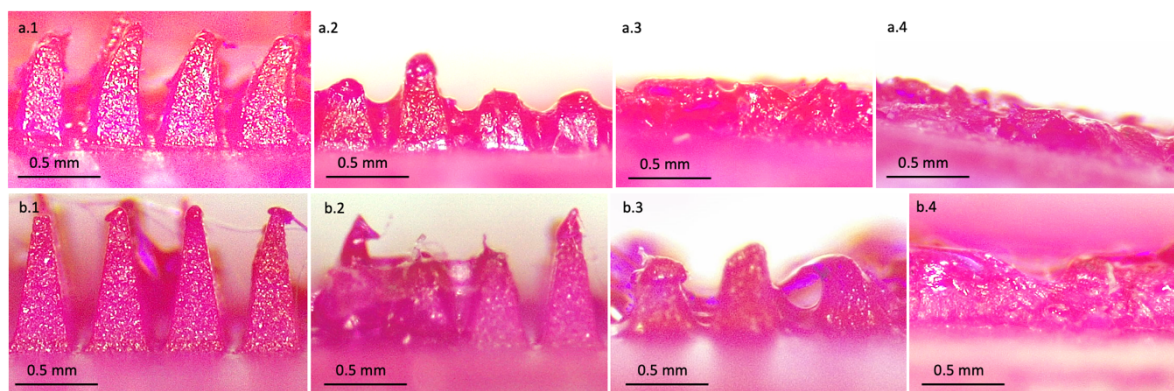


Fig. 8. Ex vivo dissolution study, investigated by optical microscopy. a) Study of RBTF-TDMNs dissolution time in the skin: 1 min (a.1), 3 min (a.2), 5 min (a.3), 10 min (a.4). Scale bar 0.5 mm; magnification 35x. b)

Study of RB-TDMNs dissolution time in the skin: 1 min (b.1), 3 min (b.2), 5 min (b.3), 10 min (b.4). Scale bar 0.5 mm; magnification 35x.

RBTF-TDMNs were entirely dissolved after 5 minutes (Fig. 8 a.3), with a bending already visible after 1 minute (Fig. 8 a.1). In contrast, RB-TDMNs were wholly liquefied after 10 minutes, with a partial dissolution and decreased MN height after 3 minutes (Fig. 8 a.2). Due to the high water-solubility of the PVA and PVP, both the TDMNs could dissolve very quickly and thoroughly. Furthermore, the presence of RBTF appeared to decrease the dissolution time of the formulation. Surfactants, represented by the SpanTM 80, play an essential role in formulating dissolving MNs. Considering the water-rich environment characterising the dermis, it is likely that SpanTM 80 promoted the wetting and spreading of the dermal interstitial fluid along and into the polymeric matrix, thus enhancing the water access into the MNs and finally increasing the overall dissolution rate [42].

3.7 *Ex vivo* dermatokinetic study

To assess the effectiveness of TDMNs as an RBTF delivery platform in melanoma management, we finally investigated ex-vivo the percutaneous absorption of RB when formulated as a transfersomal dispersion (RBTF), integrated free into TDMNs (RB-TDMNs) and incorporated as RBTF into TDMNs (RBTF-TDMNs).

RBTF-TDMNs were designed to assist the surgical management of early-stage melanoma, mainly when it affects anatomical locations where the surgical eradication is more complex and not convenient for the patient in terms of the quality of life. Melanoma at early stages (0, I and II) extends through the epidermis and further into the dermis in case of disease at stage II (American Cancer SocietyTM, 2019). Thus, in the dermatokinetic study, we focused on the RB amount accumulated in the epidermis (SC and viable epidermis) and dermis following the application of the formulations to the skin's outer surface, using a Franz diffusion cell set-up. Franz cells are widely used to study the percutaneous absorption of topically applied drugs, and several studies in the literature have highlighted their value in analysing dermal drug delivery [51–53]. Furthermore, Franz cells have been used to assess the drug delivery utilising ex-vivo skin of varying thickness as a diffusional barrier [54]. Herein, we chose porcine skin as a model diffusional barrier due to its similar morphology and function to its human counterpart; notably, we employed full-thickness porcine skin instead of dermatomed to prevent any alteration of the skin biomechanical properties, which may cause an overpenetration of TDMNs into the skin [55,56]. The RB amount delivered in the

porcine's skin epidermis and dermis, and the corresponding delivery efficiencies are depicted in Fig. 9.

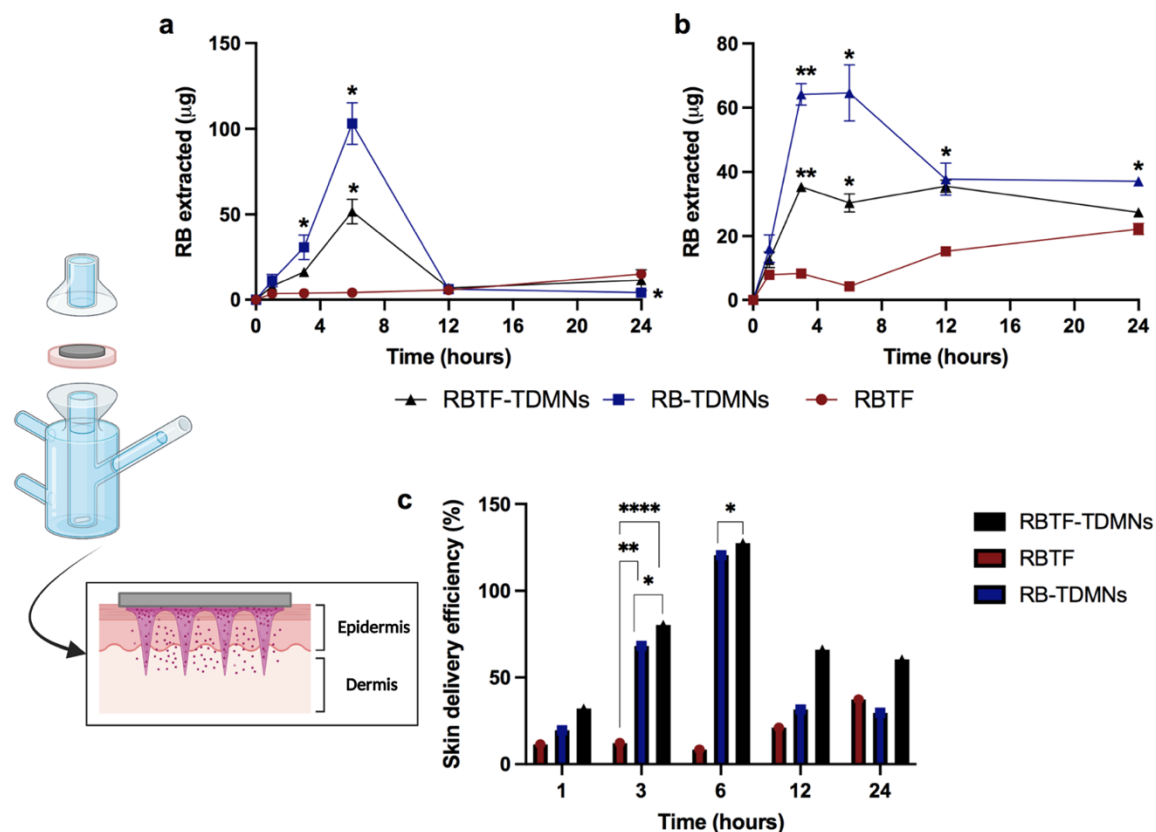


Fig. 9. Ex-vivo dermatokinetic study. Amount of RB extracted from (a) epidermis and (b) dermis of excised neonatal porcine skin. (c) The delivery efficiency of RB delivered into the skin over 24 hours (values represent means $\pm$ SEM, $n=3$). **** p value <0.0001 ; ** p value <0.01 ; * p value <0.05 .

The skin treated with RBTF dispersion delivered a significantly lower RB amount than both the TDMNs formulations, clearly demonstrating the value of TDMNs as an epidermal and dermal delivery system. The application of RBTF dispersion onto the skin's surface implies that the vesicles migrate through the different layers of the tissue to reach the target site; the presence of the surfactant in the formulation of RBTF makes the vesicles flexible and able to withstand this process. Even though TF show better performances than liposomes, the predecessors, the permeation mechanism is still controversial in the literature. TF are supposed to squeeze and pass-through skin pores without losing their integrity; however, other processes are also contemplated, including the structural modification of the intracellular lipid matrix and the vesicle's adsorption to and fusion with the SC [57,58]. Therefore, part of the RBTF may lose its main structural characteristics during the permeation process or leak, causing undesired drug waste and reducing the APIs delivered to the dermis. Moreover, formulating RB in TF is critical to improving cellular uptake and therapeutic

efficiency; thus, the delivery at the target site of intact RBTF is crucial in this project. On the opposite, as shown in section 3.5, both the TDMNs penetrated the skin to a depth of around 400 μm , thus physically allowing the encapsulated cargo to reach the target site, circumventing the migration process limits. Moreover, as anticipated in section 3.3, the current TDMNs are equipped with a solid PLA baseplate in contact with the skin surface that provides an occlusive dressing minimising the transepidermal water loss and contributing to the drug permeation through the tissue [59,60].

Once experimentally proved the role of the trilayer dissolving MNs technology in cutaneous drug delivery, we evaluated the different RB permeation profiles from the RB-TDMNs and RBTF-TDMNs. RB-TDMNs showed a similar profile of intradermal delivery capability as RBTF-TDMNs but provided a faster permeation. During the first 6 h, the RB permeation from RB-TDMNs was significantly higher than RBTF-TDMNs into both epidermis (p value <0.05) and dermis layers (p value <0.05). However, regarding skin delivery efficiency, RBTF-TDMNs deposited an RB amount higher than RB-TDMNs into the skin strata at 6h. The results suggest that the inclusion of RBTF into RBTF-TDMNs allowed a higher deposition and better-equilibrated distribution of the drug cargo within the epidermis and dermis compared to RB-TDMNs, improving the delivery efficiency and avoiding drug waste. The dermal delivery profile further suggests that the inclusion of RBTF in the polymer matrix increased the residence time of RB into the dermis compared to RB-TDMNs. Indeed, it is essential to highlight a dramatic decrease in RB percentage delivery from 12 h to 24 h. This is possibly due to RB permeation across the skin and into the receptor compartment increasing over the dermatokinetic studies.

The findings reported above may be explained considering the physical-chemical profile of the RB molecule. RB disodium salt is highly water-soluble (100 g/L) and amphiphilic, with a significant tendency for hydrophilicity. TDMNs rapidly dissolved in the water-rich skin surrounding (section 3.6), releasing the encapsulated cargo. It is likely that free RB, once bypassed the SC barrier thanks to RB-TDMNs, has quickly migrated to the deepest skin layers containing more water than the upper ones due to the skin hydration gradient [60]. Nevertheless, the encapsulation of RB in TF enhances its hydrophobicity [61] and consequently the affinity for lipid-rich environments, thus slowing the permeation rate from the epidermis to the dermis and increasing the overall RB residence time in the skin layers. This hypothesis is supported by analysing the skin permeation profiles of water-soluble and hydrophobic drugs released by analogues dissolving MNs. Recently, the drug delivery efficiency of the same dissolving MNs containing, in one case, ibuprofen sodium (water-soluble) and, in the second case, itraconazole (hydrophobic) was compared [42]. The study demonstrated

the higher permeation of ibuprofen sodium across the skin compared to itraconazole, which instead deposited into the skin. Similarly, the authors attributed the poor skin permeation of itraconazole to its low aqueous solubility.

To conclude, the dermatokinetic study showed that formulating RB in RBTF-TDMNs was an effective strategy to improve drug delivery to the skin layers affected by early-stage melanoma. As already stated, it is essential to remind that RBTF is also fundamental to promoting the interaction of RB with melanoma cancer cells, as proved in our previous research work [14].

4. Conclusion

The current work aimed to investigate the ability of TDMNs to act as an RB-loaded TF intradermal delivery system as an adjuvant tool in early-stage melanoma surgical management. TDMNs can play an essential role in avoiding a late recurrence of this disease, as they painlessly pierce the skin releasing the active cargo directly to the target site. We demonstrated that the novel RBTF-TDMNs were strong enough to penetrate the skin. They rapidly dissolved in the tissue fluid to release RBTF, which were successfully deposited in the area affected by melanoma at an early stage. Notably, RBTF-TDMNs significantly increased the amount of RB deposited in the skin compared to the RBTF dispersion; more importantly, the delivery efficiency of RBTF-TDMNs was higher than the TDMNs loaded with the free drug. The integration into the polymer matrix and the release process did not alter the RBTF structural characteristics essential to preserving the advantages of nanotechnology. The outcomes of this research strongly encourage considering RBTF-TDMNs a valuable tool to fight melanoma cancer. Even if this investigation is focused on intradermal delivery, *in vivo* testing would be of great value to better understand a realistic condition. To the best of our knowledge, the pharmacokinetics of RB, alone and when associated with lipid carriers, are not deeply documented in the literature, and it's necessary to go through this topic since it is highly employed in the biomedical field, specifically in cancer therapy. In this regard, it is our goal to elucidate the *in vivo* behaviour of pure RB, RBTF and RBTF-TDMNs in future investigations.

CRediT authorship contribution statement

Sara Demartis: *Conceptualization, Investigation, Validation, Formal analysis, Writing – original draft, Visualization.* **Qonita Kurnia Anjani:** *Methodology, Validation, Formal analysis, Investigation, Writing – review & editing.* **Fabiana Volpe-Zanutto:** *Methodology, Investigation, Writing – review & editing.* **Alejandro J.**

Paredes: Conceptualization, Writing – review & editing. **Subrin A. Jahan:** Investigation. **Lalitkumar K. Vora:** Writing – review & editing. **Ryan F. Donnelly:** Conceptualization, Resources, Writing – review & editing, Supervision. **Elisabetta Gavini:** Conceptualization, Writing – review & editing, Supervision.

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Research Article

Rose Bengal Transfersomes targeted lymphatic delivery mediated by Hollow Microneedles to treat metastatic melanoma: a proof of concept.

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This section of the thesis reports an *in vivo* study evaluating the use of Hollow Microneedles to favour the intradermal injection of Rose Bengal loaded Transfersomes aiming to a lymphatic targeted delivery. The study has been performed in the week of 15-22 January 2023; therefore, only preliminary results will be reported. The investigation has been performed in collaboration with Prof. Ryan Donnelly from Queen's University Belfast.

1. Introduction.

Nowadays, malignant melanoma represents a severe public health problem. Melanoma is the skin cancer form with the lowest incidence rate but highest mortality due to its ability to metastasise rapidly to the regional lymph nodes. Luckily, most melanoma patients are diagnosed when the disease is at its early stages (stages I and II), which is still localised to the outmost skin layers. The diagnosis of melanoma at advanced stages is not usual, and campaigns to prevent the rise of diseases have been progressively decreasing the incidence [1]. Patients with localised disease often recover entirely after the surgical resection of the lesion; still, the prognosis of patients with advanced disease depends on the cancer stage, based on the evaluation for metastases. Patients with primary melanoma thicker than 1 mm undertake intraoperative lymphatic mapping and biopsy to recognise the deposition of microscopic metastatic cells [2]. The lymphatic system's role in tumour metastasis has been increasingly investigated through studies of murine and human cancers; mainly, malignant cells can be observed within lymphatic vessels, suggesting its leading role in cancer spread [3]. Targeted therapy and immunotherapy are the pillars of the treatments available for metastatic melanoma. However, despite the evident progress in increasing patients' life expectancy, the resolute outcome of these approaches remains limited. In this regard, the clinical evolution and drug efficacy in melanoma patients are rarely predictable; on the other side, induced resistance to pharmacological therapies and the incidence of adverse side effects often occur. Therefore, the development of advanced treatments remains a priority [1,4]. Viewing the role of the lymphatic vessels and, in general, of the lymphatic system in favouring the systemic spreading of cancer, much research is focusing on its target. Notably, lymphatic delivery based on nanotechnology revealed high potential benefits for advanced melanoma management [5].

Rose Bengal (RB) is an effective anti-melanoma agent that is currently involved in clinical trials for the treatment of metastatic cutaneous melanoma under the name of PV-10® (Provectus Biopharmaceuticals, INC.). RB's current administration route is the intratumoral injection due to its short half-life and poor lipophilicity. However, the intralesional injection of RB has been reported as extremely painful, and multiple administrations are needed to reach the therapeutic dose [6]. Finally, due to RB photosensitising properties and limited cancer selectivity, a case of severe systemic phototoxic reactions have been reported [7]. Thus, a delivery system able to enhance the RB selectivity without causing further discomfort to the patient is demanded.

For this purpose, we previously developed and characterised RB loaded transfersomes (RBTF) dispersion in the nanosized range, which significantly decreased melanoma cell growth compared to the free drug's solution and enhanced the selectivity towards malignant than healthy cells [8]. Based on this evidence and the crucial role ascribed to the lymphatic system in melanoma progression, we evaluated herein whether RBTF dispersion had the potential to properly accumulate in the lymphatic system, particularly into the tumour-draining lymph nodes.

An appropriate drug administration route is mandatory to ensure efficient lymphatic delivery. The percutaneous route represents a non-invasive and valuable way to target the lymphatic system, and it has been reported to increase delivery efficiency compared to other administration routes. The intradermal space is highly perfused with vascular and lymph capillary networks, in which cells, interstitial fluid, and large particles circulate and drain to the lymph nodes. Regarding nanoparticles (NPs) uptake, evidence reported that NPs in the range of 10–100 nm are taken up effectively by the lymphatic system. Conversely, NPs >100 nm are retained at the administration site, and NPs <10 nm are taken up by blood capillaries. Even though this phenomenon is essential to favour the lymphatic targeting by NPs, the migration of NPs through the whole skin up to reaching and being absorbed by lymphatic vessels is a utopia considering the excellent barrier properties of the stratum corneum (SC) [9–12]. The painless intradermal deposition of NPs cargo would bypass this issue; hollow microneedles (HMNs) have been demonstrated to centre this need.

HMNs are a microscopic version of the conventional intradermic injection, which delivers drugs through the drive of liquid pressure. Considering the micrometric size of both their length and diameter, the fabrication of HMNs is expensive, requiring significant technological resources. The materials for preparing hollow MNs are commonly inorganic, such as metals, silicon, ceramics, and glass. Nevertheless, compared to other MNs types, like dissolving MNs, HMNs have higher drug delivery capacity, allowing continuous drug release with accurate dosing while increasing therapeutic adherence compared to the intradermic injection [13,14].

The study herein presented mainly aimed to evaluate, through an *in vivo* study, if the HMNs technology could facilitate the intradermal deposition of RBTF dispersion, thus enabling their uptake by the lymphatic system in view of potential metastatic melanoma treatment. Furthermore, the pharmacokinetic and tissue distribution study of RBTF dispersion and RB aqueous solution was evaluated via HMNs and intravenous injection.

2. Methodology.

Briefly, RB solution and RBTF dispersion were prepared (RB = 4 mg/mL). RBTF dispersion was obtained following the preparation method previously reported [15].

The *in vivo* pharmacokinetic and biodistribution studies were approved by the Committee of the Biological Services Unit, Queen's University Belfast. The work was carried out under Project License PPL 2903 and Personal Licenses PIL 2127 and 2059. All experiments were conducted based on the policy of the Federation of European Laboratory Animal Science Associations and the European Convention for the protection of Vertebrate Animals used for experimental and other scientific purposes, implementing the principles of the 3Rs (replacement, reduction and refinement). Healthy female Sprague–Dawley rats with an average weight of 280 mg and aged between 10 and 12 weeks were acclimatised to the laboratory conditions for one week before the commencement of the experiments. The animals were divided into six cohorts, resumed in table 1.

Table 1. Cohort tested in the *in vivo* study.

<i>Cohort</i>	<i>Formulation</i>	<i>Dose</i>	<i>Route</i>	<i>Blood sampling</i>	<i>Study endpoint</i>
1	RB solution	4.0 mg/kg	i.v. injection	15 min 30 min 45 min 60 min	2 hours
2	RB transfersomes				
3	RB solution	4.8 mg/kg	HMNs injection		
4	RB transfersomes				
5	RB solution				
6	RB transfersomes				
					24 hours

i.v.: intravenous injection; *HMNs*: hollow microneedles injection.

The first cohort received a single intravenous injection of an aqueous solution for an RB dosage of 4.0 mg/kg. The animals in the second cohort were administrated with a single intravenous

injection of an RBTF dispersion for an RB dosage of 4.0 mg/kg. Cohorts three and five received a single administration of RB solution (RB = 4.8 mg/kg) via HMNs injection; finally, cohorts four and six received a single administration of RBTF dispersion (RB = 4.8 mg/kg) via HMNs injection. HMNs used in this investigation consisted of three needles with a length of 600 μm (Nanosoft® Microneedles, FillMed Laboratories, France). The rats were firstly sedated using gaseous anaesthesia (2–4% isoflurane in oxygen). For cohorts three to six, the hair from their backs was removed using an electric hair clipper and Louis marcel hair removal cream (Sodalis Srl, Milano, Italy). MNs were then applied onto the backs of the rats, and the formulations were injected with the aid of a syringe directly connected to the HMNs device. To ensure the MN arrays remained securely in place, a small piece of Tegaderm™ (3 M, St Paul, Minnesota, USA), was placed on top of the MNs, and Micropore™ tape (3 M UK Plc, Bracknell, Berkshire, UK) was used to cover the back of the rats. For the plasma pharmacokinetics, blood samples were obtained via tail vein bleeds at predetermined time intervals: 15 min, 30 min, 45 min and 60 min, with a maximum of 200 μL collected at each sampling time point into heparinised tubes. For the lymph pharmacokinetic and biodistribution study of RB solution and RBTF administered by intravenous injection, cohorts one and two were sacrificed after two hours from the drug administration and the brain, lungs, liver, kidneys, and lymph nodes (axillary and popliteal) have been harvested for histological analysis. For the lymph pharmacokinetic and biodistribution study of RB solution and RBTF administered by HMNs, cohorts three and four were sacrificed after two hours from the drug administration, while cohorts five and six were sacrificed after twenty-four hours from the drug administration; the brain, lungs, liver, kidneys, and lymph nodes (axillary and popliteal) have been harvested for histological analysis in both cases.

Finally, the blood samples and the organs were processed to proceed to the qualitative and quantitative analyses. Blood samples were submitted to an RB extraction process to quantify the analyte in the systemic circulation at the predetermined time points and evaluate the pharmacokinetic profile. The harvested organs, namely the brain, lungs, kidneys, liver, and axillary and popliteal lymph nodes, were analysed through multiphoton microscopy to estimate the biodistribution of RB. Moreover, the lymph nodes were further processed to extract the analyte and quantify the RB amount accumulated.

3. Preliminary results.

In this investigation, an RB solution and the RBTF dispersion ranging in 50-100 nm size were evaluated for their ability to target the lymphatic system. Two administration routes were tested that is (i) intravenous injection and (ii) intradermal injection mediated by HMNs. The pharmacokinetic and biodistribution of RB were studied over 2 and 24 hours for the intravenous and intradermal injection, respectively. In the case of intravenous injection, the dose of administered RB was 4.0 mg/kg but 4.8 mg/kg in the case of intradermal injection. Indeed, evidence reported that the bioavailability from HMNs array is approximately 80% of the initial administered dose, meaning that a 4 mg/kg is estimated to be delivered *in vivo* in the rats.

Regarding organs, apart from lymph nodes, the brain, lungs, and kidneys were harvested as well. The rationale of this procedure relies on the fact that those organs are preferentially metastasised by malignant melanoma. Therefore, the evaluation of the biodistribution of RB is essential to better investigate its potential as a novel treatment for this disease.

Given the recent performance of the *in vivo* study (15-22 January 2023), only preliminary qualitative results are available for the current report. The procedure of RBTF dispersion intradermal injection mediated by HMNs is illustrated in Figure 1.

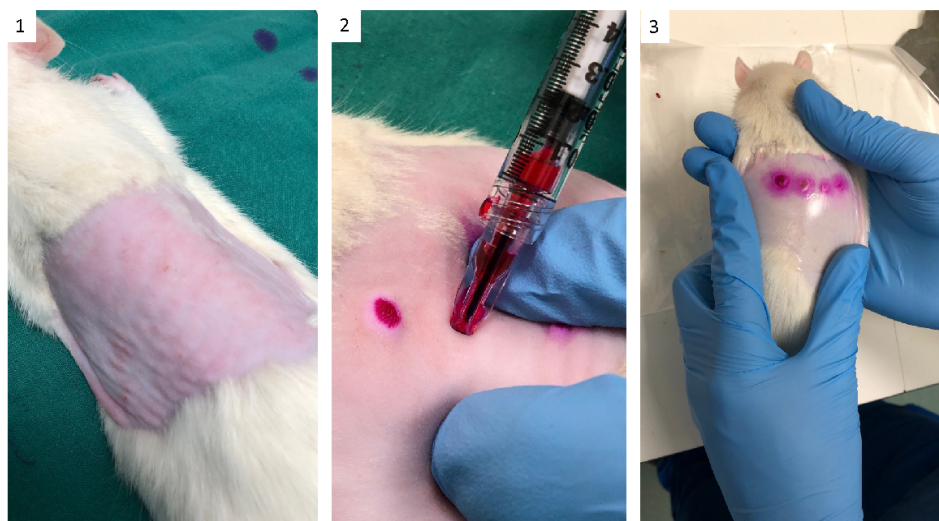


Fig. 1. Administration of RBTF dispersion by HMNs-mediated intradermal injection. 1. Shaved rat back. 2. Intradermal injections RBTF dispersion via HMNs in the rat back. 3. View of the total intradermal injections.

The lymph nodes of all six cohorts were analysed. At the end point of the *in vivo* study, lymph nodes were harvested and visually investigated to estimate the RB amount delivered qualitatively. Based on the qualitative analysis (figure 2) and preliminary quantitative analysis (data not reported), results suggest that the highest RB lymphatic delivery was obtained when RBTF dispersion was administered via intradermal injection mediated by HMNs after two hours from the administration (cohort 4).

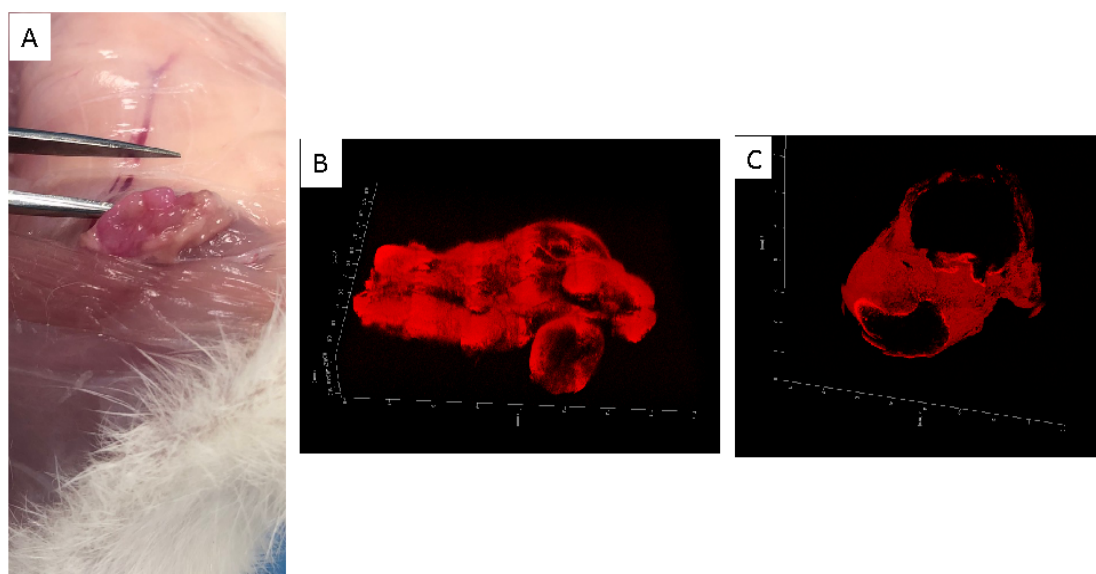


Fig 2. Representative axillary's lymph node of cohort 4. A. Appearance of the lymph node at the endpoint of the study. B. Micrograph of the lymph node in figure 2. A acquired by multiphoton microscopy. C. Micrograph of a representative lymph node of cohort 3 (RB solution, HMNs injection, 2 hours) acquired by the multiphoton microscopy.

The elaboration of the data acquired during the study will allow us to confirm or dismiss this preliminary outcome. The data to be elaborated include:

- I. The concentration of RB in blood samples at the predetermined sampling points by quantitative determination;
- II. The concentration of RB in lymph nodes by quantitative determination;
- III. The concentration of RB in other organs harvested, namely the brain, lungs, kidneys and liver, by qualitative determination.

4. Conclusion and future perspectives.

Through the current chapter, the project titled "Transfersomes: The Newly Developed Rose Bengal Delivery System to Target and Manage Cutaneous And Metastatic Melanoma" was presented and discussed, starting from the rationale at the basis of its development up to the conclusion.

Undoubtedly, malignant melanoma is a serious public health concern, and the scientific community's interest in this theme is mandatory. In the last decades, a pink-coloured molecule initially synthesised as a textile dye, Rose Bengal (RB), has been actively investigated for its particular biomedical benefits. Among these, the high intrinsic cytotoxicity towards malignant melanoma cell lines gained much attention, and several clinical trials have proved its potential. However, the

characteristics of high water solubility and the ability to induce phototoxic reactions decrease its beneficial therapeutic effect. To prevent this issue, RB was herein loaded in an ultradeformable vesicle dispersion, named transfersomes (TF) and administered through the percutaneous route, which facilitates the exposure of melanoma lesions to therapeutics. RB-loaded TF proved to effectively promote the cytotoxicity of RB selectively to cancer cells, to control its release, to enhance its permeation through the outmost layers of skin and to protect the drug from photodegradation. Nevertheless, a deeper deposition is needed in the case of treating lesions infiltrating the dermis. This goal was achieved by combining RBTF with micro-needling technology. Dissolving polymeric microneedles were tested to provide an intradermal RBTF release. They were demonstrated to efficiently target the RBTF cargo in the dermis, resulting in a promising tool to manage melanoma at its earliest stages. Still, dissolving polymeric microneedles' relatively low loading capacity prevented their utility in treating advanced melanoma at the metastatic stage. Thus, a higher dosage of RBTF was intradermally injected following the application of hollow microneedles and evaluated in an *in vivo* study, representing the conclusion of this project. The data from the *in vivo* study still need to be elaborated, but promising outcomes emerged. The future perspective includes investigating in detail the pharmacokinetic and biodistribution properties of RBTF dispersion; finally, a study elucidating the biological and pharmacological behaviour of RBTF vesicles when in contact with melanoma cells will be performed.

CRedit authorship contribution statement

Sara Demartis: *Conceptualization, Investigation, Validation, Formal analysis.* **Elisabetta Gavini:** *Conceptualization, Methodology, Supervision.* **Qonita Kurnia Anjani:** *Methodology, Validation, Formal analysis, Investigation.* **Fabiana Volpe-Zanutto:** *Methodology, Validation, Formal Analysis, Investigation.* **Akhmal B. Sabri:** *Methodology, Investigation.* **Aaron Hutton:** *Methodology, Investigation.* **Ryan F. Donnelly:** *Resources.* **Giovanna Rassu:** *Conceptualization, Methodology, Supervision.*

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Chapter III.

Biodegradable 3D-printed reservoir-type implants for the sustained release of Rose Bengal: application to local cancer therapy.

Research Article

Biodegradable 3D-printed reservoir-type implants for the sustained release of Rose Bengal: application to local cancer therapy.

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1. Introduction

According to the World Health Organization (WHO), cancer is a leading cause of death worldwide, accounting for almost one in six deaths. The International Agency for Cancer Research (IARC) reported that 19.3 million new cancer cases were diagnosed in 2020, and 9.96 million people died from the same disease. These numbers are estimated to increase, during the following 20 years, to 30.2 and 16.3 million, respectively (GLOBOCAN 2020). Although the remarkable progress in the last decades, previous statistics affirm that new and efficient therapeutic approaches are urgently needed. Cancer treatment is a complex and challenging undertaking. Depending on the stage and location, surgical resection is the first-line therapy but cannot be applied in all cases. Moreover, microscopic cancer lesions may persist after mass removal and require coupling with additional treatment, such as radiotherapy, commonly reporting long-term toxicity and limited efficacy. Conventional chemotherapy is often proposed as an adjuvant to surgical resection and in case of unresectable lesions. Still, this approach often presented severe side effects due to unspecific drug delivery and difficulty achieving the appropriate drug dosage to the tumour site [1]. Those limits are commonly related to the narrow therapeutic window of chemotherapeutics or the poor biopharmaceutical profile [2].

The implantable devices specifically designed for *in situ* cancer therapy represent an efficient way to reach the therapeutic dose while minimising the systemic toxicity of the anticancer drug by reducing its concentration in blood circulation; indeed, they provide precise spatial control of the drug release preventing damage to healthy cells and increasing the overall survival rate. Moreover, implants can avoid some drug biopharmaceutical issues, protecting the molecule from degradation or clearance until the release [2,3]. Among the implantable devices reported in the literature, 3D-printed implants have increasingly attracted local chemotherapy attention since a wide range of parameters can be adjusted to obtain an implantable device with the desired structural characteristics and release profile, thus enhancing the precision of the therapy [4]. Wang *et al.* designed 3D-printed poly L-lactic acid (PLLA) implants as an individualised local osteosarcoma therapy, testing the co-loading of doxorubicin, ifosfamide, methotrexate and cisplatin [4]. The authors proposed two different shapes, spherical and cylindrical, to discover that the drug loading of the spherical implant was much greater than the cylindrical one highlighting the importance of this parameter in the encapsulation efficiency of 3D-printed devices. The biodegradability, *in vitro* cytotoxicity and blood compatibility, *in vivo* toxicity and sensitisation tests have proved loaded implants' biocompatibility and pharmaceutical properties. Furthermore, the *in vivo* drug release

kinetic from the spherical implant demonstrated that all the drugs reached the plasma peak value after two weeks. The sustained release could last twelve weeks, showing a higher drug concentration in the target tissue than in the whole blood. Finally, the research showed that local chemotherapy by the 3D-printed device was more effective than traditional chemotherapy. Similarly, Yang et al. developed a drug delivery implant for breast cancer therapy to suppress tumour growth and reduce pulmonary metastasis by combining 5-fluorouracil (5-FU) and NVP-BEZ235 [5]. The authors used the electro-hydrodynamic jet (E-jet) 3D-printing technique to construct loaded PLGA scaffolds with 50, 100 and 150 μm aperture sizes. The drug release occurred first through a burst during the first week, followed by a slow release and a final fast stage, releasing the total drug within 30 days; this profile has been attributed to the complex degradation process of PLGA. The aperture size affected the release rate, accelerating with a larger size. The *in vivo* studies emerge that, compared to the repeated systemic injections of chemotherapeutics, the system significantly reduced the drug dosages needed while maintaining effectiveness and the therapeutic drug levels at the tumour site for an extended time. 3D-printed scaffolds are widely reported in the literature to assist cancer therapy. The well-known and model chemotherapeutic drug doxorubicin (DOX) was also included in scaffolds for breast and prostate cancer application [6,7]. In the first case, Dang et al. produced a poly(ϵ -caprolactone) device with macroscale pores of 300–500 μm in size to obtain a drug loading efficacy of 90%. Compared to a single intravenous injection of 40 μg DOX, implantation of scaffolds containing 2–8 μg of DOX after tumour removal shows reduced cytotoxicity and cancer recurrence correlated with a lower metastasis progression in 28 days of treatment. In the case of prostate cancer investigation, Ahangar et al. developed and tested *in vitro* nanoporous discoid scaffold intended to deliver DOX to the bone metastases that could also potentially serve as a bone substitute. Nonetheless, even non-conventional anticancer drugs were loaded in 3D-printed scaffolds, including curcumin. Li et al. proposed a curcumin-loaded biodegradable and flexible scaffold for intracranial therapy of GBM [7]. The scaffold was printed by extruding polycaprolactone filaments and was evaluated *in vitro* to establish biodegradation and anticancer activity. The authors investigated the influence of various parameters, such as geometric models, the pore shapes, and thicknesses of the final implant. The properties can be modified by 3D printing to perfectly match the implant with the size and shape of the tumour cavity.

Rose Bengal (RB) is a xanthenic molecule with a proven anticancer activity towards several cell lines, including colon, breast, oral, ovarian, pancreas, gastric, head and melanoma cancers [8–14]. RB may act through two mechanisms of action: its photo-sonosensitizing properties and intrinsic

cytotoxicity. Indeed, the presence of the xanthenic ring in the RB structure is responsible for its activation when triggered by light or ultrasounds stimuli, which consequently permits its application in photodynamic and sonodynamic therapy [15]. At the same time, the parallel intrinsic cytotoxicity of RB has been evidenced; RB is likely to preferentially accumulate in malignant cell lysosomes to cause cell lysis finally. This subsequently triggers a second response based on an immune reaction [16]. Besides, RB does not lack shortcomings. The reduced half-life (30 minutes) and the induction of severe systemic toxicity, related to its photosensitising properties, limit RB application [17,18]. To date, the intralesional injection of an RB aqueous solution is the only administration route, in case of cancer treatments, undertaken a clinical trial. However, repeated administrations are required to ensure the RB therapeutic window, exposing patients to painful procedures and involving constant medical assistance. Thus, the development of a long-term controlled RB delivery system is deemed.

In this investigation, we propose the development and characterisation of a reservoir-type implantable device filled with RB polymer-based formulations for the sustained release of RB in the localised therapy of cancer. The unloaded implantable device herein presented was projected, developed, and characterised by the research group of Dr Eneko Larraneta (School of Pharmacy, Queen's University Belfast, UK) [19]. The study led to the successful fabrication of a biodegradable 3D-printed reservoir implant, where a porous membrane could sustain the release of the model drug "tetracycline" for four weeks. Also, the resulting implant showed excellent cytocompatibility when tested on human umbilical vein endothelial cells. Given its biocompatibility properties, biodegradability and controlled release of drugs, the cited implant perfectly matches the need to obtain an RB delivery system that could spatially and temporarily control its release while maintaining high patient compliance. Indeed, the RB-loaded implantable devices are thought to be surgically implanted near the tumour site without requiring a surgical removal at the end of the therapy; furthermore, their application can be envisioned as an adjuvant therapeutic after the surgical resection of the primary tumour or as self-standing treatment.

2. Materials and Methods

2.1 Materials

Rose Bengal (RB) was of ≥ 95 % purity (Sigma-Aldrich, Dorset, UK). Poly(caprolactone) (PCL) polymers CAPA™ 6506 (MW = 50 kDa) and CAPA™ 2054 (MW = 550 Da) were obtained from Ingevity

(South Carolina, USA). Granulated Poly(lactic acid) (PLA) polymers Ingeo™ 3D850 (MW = 190 kDa) and Ingeo™ 4043D (MW = 200 kDa) were obtained from NatureWorks (Minnesota, MN, USA). Dichloromethane (DCM) was issued from Merck (Darmstadt, Germany). Glycerol was used of $\geq 98\%$ purity (Cruciani Prodotti Crua, Rome, Italy). Polymer A was a polysaccharide cationic polymer; polymer B was a non-swellable hydrosoluble polymer.

2.2 Fabrication and filling of the implantable device.

The reservoir-type implants were fabricated in the laboratories of Queen's University Belfast and consist of hollow cylinders presenting in one extremity a permeable membrane controlling the drug release and in the second extremity an impermeable covering (seal). Two types of polymers were used for the fabrication, polylactic acid (PLA) and poly-caprolactone (PCL), with different molecular weights: for the permeable membrane PLA (PM = 190 kDa) and PCL (PM = 550 Da) were used in a 50:50 ratio. PLA with a PM of 160 KDa was used to obtain the hollow cylinder, while the seal consists of PCL with a PM of 50 KDa. The seal is applied after filling the implant with the pharmacologically active formulation. The implant was obtained through the 3D printing technique of fused deposition modeling, and it was 3D-printed directly on the top of the permeable membrane (Ultimaker 3, 3D-printer). Finally, implants were filled with different RB formulations (section 2.3) and sealed with molten PCL at high molecular weight. The fabrication process of the implantable device is schematised in figure 1, while the full explanation of its design, development and characterisation is described in the original article [19].

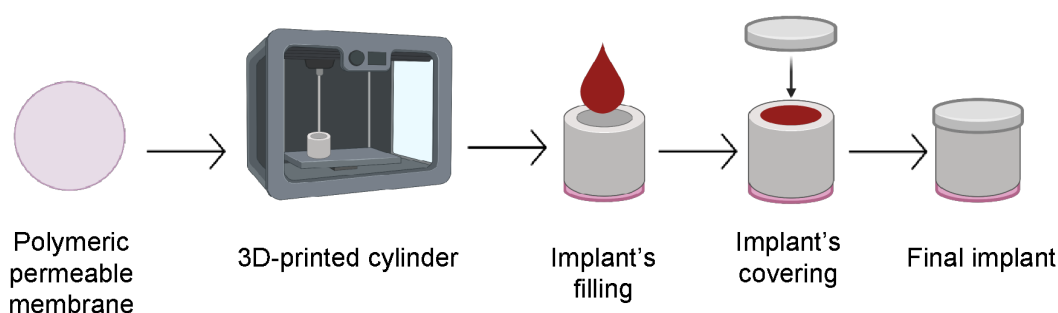


Fig. 1. Fabrication process of the implantable device.

2.3 RB long-term chemical stability.

The long-term chemical stability of RB was evaluated in a phosphate buffer solution at pH 7.4, similar to the physiological pH. The test was performed by solubilising RB in phosphate buffer (5% w/v) and incubating it at 37°C for 22 weeks, protected from light. At each predetermined time point, the

absorbance value (Abs) at RB maximum absorption wavelength in the medium (549 nm) was recorded and used to assess the chemical stability of the drug. The experiment was performed in triplicate and analysing each solution in triplicate (n=9).

2.4 Preformulation study of RB filling formulations.

A preformulation study aiming to select the leader filling was performed to identify the optimal process and formulation parameters. The type of formulation prepared were water-based or anhydrous pastes, viscous solutions, physical mixtures of polymer and drug powders, and anhydrous scaffolds obtained by evaporation or lyophilization processes. RB formulations were of polymeric nature. Herein, two polymers have been tested: a polysaccharide cationic polymer (A) and a hydrosoluble polymer (B). Table 1 resumes the formulations tested.

Table 1. RB formulation tested as implant fillings.

<i>Abbrev.</i>	<i>Implant</i>	<i>Polymer</i>	<i>Formulation</i>	<i>Technique</i>	<i>RB (mg) ¹</i>
RB	I-RB	-	RB powder	-	2.33 ± 0.51
O	I-O	-	Water-based paste	homogenisation	36.53 ± 5.25
A1	I-A1	A	Scaffold	Freeze-drying	0.59
A2	I-A2	A	Anhydrous paste	Blending	1.49
A3	I-A3	A	Powder mix	Blending	1.07
B1	I-B1	B	Scaffold	Freeze-drying	-
B2	I-B2	B	Powder mix	Blending	0.47 ± 0.04
B3	I-B3	B	Scaffold	Casting-evaporation	0.37 ± 0.09

¹ RB (mg) tested refers to the RB amount loaded into the implant. Value refers to the mean ± standard deviation of three samples. The mean value is reported when applicable.

Formulation 0 was a water-based paste of RB simply obtained by homogenising a determined amount of RB with a drop of water with a SpeedMixer® DAC 150.1 FVZ-K (Hauschild GmbH & Co. KG, Westfalen, Germany) at 3000 rpm for 5 min.

Formulations A1 and B1 were set up by freeze-drying a previously prepared polymer-based viscous aqueous solution. Briefly, RB was first solubilised in the aqueous solution of the polymer, which was subsequently cast in a glass Petri Dish (7.5 cm in diameter) and frozen overnight at -80°C. The day after, the formulation was freeze-dried for about 8 hours with Lio 5P Cinquepascale (Trezzano sul Naviglio, Italy) to obtain an anhydrous scaffold. Lastly, small 3-5 mm disks were obtained to be loaded in the final implant.

A2 was formulated by blending RB, polymer A and a humectant excipient (glycerol) in a mortar.

Formulations A3 and B2 were obtained by finely blending the polymer and RB powders in a mortar.

Finally, formulation B3 was prepared by a casting-evaporation technique starting from an RB/polymer viscous solution. Briefly, RB was first solubilised in the aqueous solution of the polymer, which was subsequently cast in a glass Petri Dish (7.5 cm in diameter) and placed in an oven (37°C, 72 hours) for complete water evaporation. The resulting scaffold was frozen for 10 min at -80 °C and ground with a mill (MF 10 basic, IKA® WERKE) for 2 min at 4000 rpm. Lastly, with a sieve with 1 mm meshes, granules higher than 1 mm were collected and used to fill the implant.

2.5 Morphological evaluation

To evaluate the morphological properties, fillings formulations and filled implants were examined by optical microscopy using an Olympus stereomicroscope (Olympus, DF PL 2X, Japan) and a scanning electron microscope (SEM) (Hitachi Tabletop™, Tokyo, Japan). The SEM analysis was carried out in low vacuum mode at a voltage of 15 kV and without sample pre-treatment.

2.6 Drug Content evaluation

To determine RB content in the filling formulations A1, B2 and B4, RB was extracted using water and/or methanol as extracting solvents. RB amount was then quantified by UV-Vis spectrophotometry and calculated using the calibration curve prepared in methanol (1-10 mg/L, $y = 121,4x - 0,0456$; $R^2 = 0,9997$) and in water (1-20 mg/L, $y = 0,1064x + 0,0053$; $R^2 = 0,9996$). Finally, the drug content (DC%) was determined by the following equation:

$$DC\% = \frac{\text{quantified RB}}{\text{formulation weight}} \times 100 \quad (1)$$

Three batches of each formulation were analysed in triplicate (n=9).

2.7 *In vitro* release studies

2.7.1 *In vitro* release of filling formulations

The *in vitro* release of filling formulations A1, and B3 was tested by separately dispersing the formulations in 10 mL of phosphate buffer (pH 7.4) and incubated at 37°C in a shaker incubator (40 rpm) (SKI 4 Shaker Incubator, Argo Lab, Carpi, Italy). At each predetermined time point, 500 µL of phosphate buffer were withdrawn and immediately replaced with the same amount of fresh medium. RB amount was then quantified by UV-Vis spectrophotometry and calculated using the calibration curve prepared in water. Finally, the cumulative RB amount released (mg) in the time function as reported in equation 2:

$$Q_n = C_n \times V_0 + \sum_{i=1}^{n-1} C_i \times V_i \quad (2)$$

Where Q_n is the cumulative amount of drug; C_n is the drug concentration of the acceptor medium at each time point; V_0 is the acceptor medium's volume; C_i is the drug concentration of the sample at each time point, and V_i is the volume of the sample collected at each time point. In the case of A1 and B2, the evaluated time points were 30 min, 1 h, 3 h, 5 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h, and 168 h; in the case of B4 were 5 min, 10 min, 15 min, 20 min e 30 min. The experiment was performed in quadruplicate for each formulation tested.

Parallely to the *in vitro* release, the dissolution rate of B4 was evaluated. Briefly, the formulation was dispersed in 10 mL of phosphate buffer (pH 7.4) and incubated at 37°C in a shaker incubator (40 rpm). At each predetermined time point (5 min, 10 min, 15 min, 20 min, 30 min), the dispersion was filtered under vacuum, and the formulation residues were frozen at a temperature of -80°C, then freeze-dried for 8 hours and lastly weighed. Finally, the weight of the formulation (mg) was plotted in the time function. The experiment tested 4 samples for each time point (n=4).

2.7.2 *In vitro* release of filled implants.

The *in vitro* release of filling formulations I-RB, I-O, I-A1, I-A2, I-A3, I-B2, and I-B3 was tested by separately immersing the implant in 10 mL of phosphate buffer (pH 7.4) and incubated at 37°C in a shaker incubator (40 rpm) ((SKI 4 Shaker Incubator, Argo Lab, Carpi, Italy). At each predetermined time point, all the 10 mL of phosphate buffer were withdrawn and immediately replaced with the same amount of fresh medium. RB amount was then quantified by UV-Vis spectrophotometry and calculated using the calibration curve prepared in water. Finally, the cumulative RB amount released (mg) in the time function as reported in equation 3:

$$Q_n = \sum_{i=1}^n C_0 \times V_0 \quad (3)$$

Where Q_n is the cumulative amount of drug; C_0 is the drug concentration of the acceptor medium at each time point; V_0 is the acceptor medium's volume. The study has been carried out for 28 weeks. The experiment was performed in quadruplicate for each implant tested.

2.7.3 *In vitro* release of the leader implant in agarose gel.

To more realistically simulate the conditions for drug release in biological tissue upon *in situ* administration, the experimental testing method illustrated in Figure 2 was used.

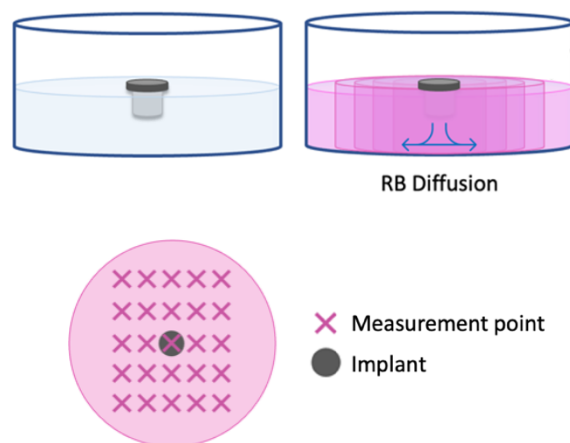


Fig. 2. Schematic illustration of the experimental set-up used to monitor RB release into agarose gels.

Ten mL of an agarose gel (0.5% w/v) were prepared by dissolving the polysaccharide in boiling water and subsequent casting into 6-well Petri dishes (well diameter = 35 mm). Upon cooling to room temperature, the gel formed. One implant was placed into one well serving as drug reservoir: at its centre. The permeable membrane was placed in contact with the gel, and the impermeable

covering was located at the surface of the agarose gel, in contact with air. The well plates were sealed with adhesive aluminium foil to minimize water evaporation and placed into a horizontal shaker at 37 °C and 40 rpm. Sink conditions were maintained throughout the experiments. A well containing only agarose gel was treated in the same way for reasons of comparison. At predetermined time points, the RB concentration at 25 positions in the agarose gel was determined with a plate-reader spectrophotometer using the calibration curve built in agarose ($y = 0.0902x + 0.2671$, $R^2 = 0.9991$).

3. Results and Discussion

An implantable device loading RB was developed to sustain its release in the localised management of cancer. Indeed, RB proved highly cytotoxic towards various malignant cell lines, but its high-water solubility and phototoxic-induced reaction represent an obstacle to its therapeutic use. Thus, a proper advanced delivery system was necessary. For this purpose, a reservoir cylindrical-shaped implant with a base diameter of 7 mm x 5 mm in height was chosen because these dimensions are comparable to the dimensions of similar devices already approved and available in the market [18]. Regarding the shape and the choice of polymer materials, the successful use of chemotherapeutics-loaded polymer cylindrical-shaped implants has been documented in the literature and proposed for local cancer therapy of liver, prostate, breast and pancreatic carcinoma [20–23]. Furthermore, the current implant is biodegradable and biocompatible, and it was tested as a sustained-release device using tetracycline as a model drug and was revealed to be suitable for the purpose [19]. Regarding the formulation of RB fillings, two biodegradable and biocompatible polymers were tested to improve the controlled release properties of the system. As a demonstration, the implants were initially filled with formulation 0, a water-based paste of RB. However, the *in vitro* release study only lasted 20 days (figure 3). Furthermore, water could pose a risk of microbial contamination that must be minimised in cancer-suffering patients.

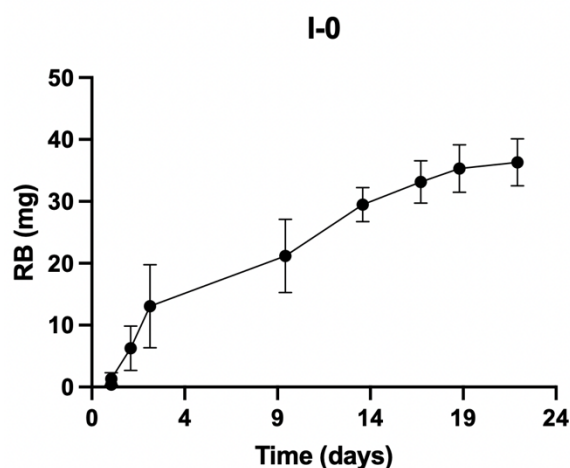


Fig. 3. In vitro release of I-0. Mean, and SD are illustrated for each time point ($n=4$).

Aiming for long-term delivery of RB, its chemical stability over a prolonged period was tested in phosphate buffer by monitoring its Abs at its maximum absorption wavelength in aqueous media, 549 nm (figure 4) [24].

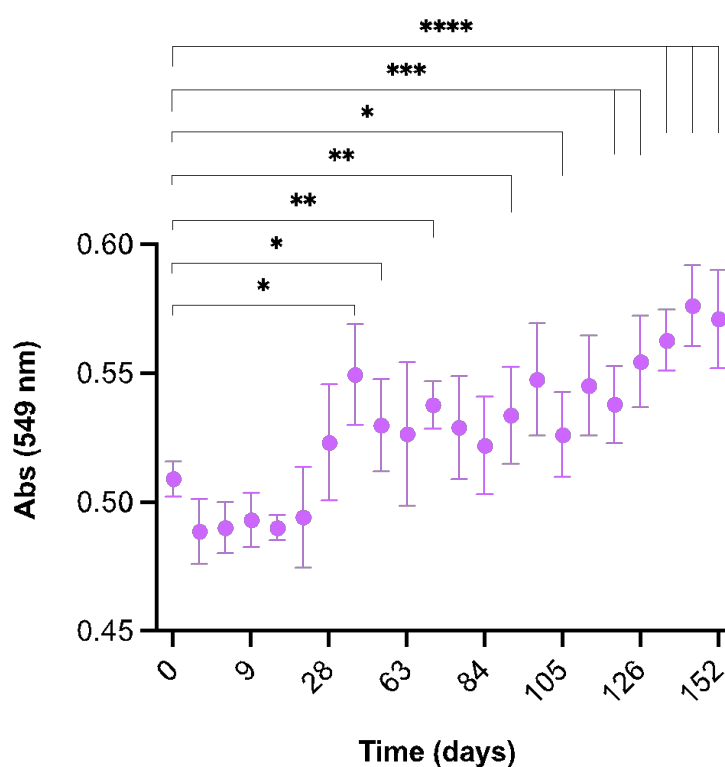


Fig. 4. Chemical stability of RB in phosphate buffer (pH 7.4). Mean, and SD are illustrated for each time point ($n=9$). * p value < 0.5 ; ** p value < 0.1 ; *** p value < 0.01 ; **** p value < 0.0001 .

The results showed that the Abs value of RB at 549 nm tends to increase significantly over time; the first significantly higher value was seen after 30 days. The tendency of RB to aggregate to dimers

in solution at high concentrations has already been reported in the literature, and it is known to affect the spectral properties of this molecule. The aggregation is particularly likely to occur in salt solution; in fact, the presence of electrolytes can affect the interaction between two molecules and, therefore, their ability to bind by means of different mechanisms. The formation of RB dimers impairs the photochemical response of this photosensitizing molecule and decreases the desired singlet oxygen generation, which is the main photodynamic therapy reaction. Nevertheless, herein, RB is intended to use for its intrinsic cytotoxic properties and not as a photosensitizing agent; therefore, this phenomenon is not likely to interfere with the therapeutic efficiency of the presented system [25, 26].

3.1 Polysaccharide cationic polymer (Polymer A).

Polymer A was evaluated in the preformulation study to obtain a long-term delivery system and chosen for its cationic charges. RB is a dye in which the charge on the chromophore is negative; thus, we hypothesised the formation of a polyelectrolyte complex between the drug and polymer A that could control the release. To favour the development of a proper filling formulation, we applied different techniques, from conventional physical blends to freeze-drying. The polyelectrolyte complex between RB and Polymer A was formed, but several adverse outcomes were observed. In the case of formulation A1 (freeze-dried scaffold), the experimental DC% accounted for 0.61 ± 0.52 , revealing a coefficient of variation of 85%. The DC% was not uniform through the scaffold, probably due to a not homogeneous polyelectrolyte complex formation (figure 5A, 5B).

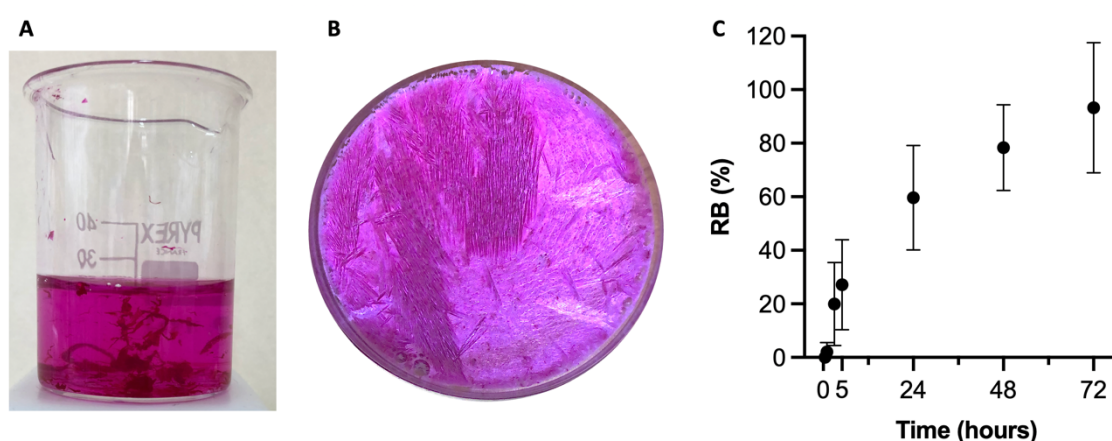


Fig. 5. Formulation A1 before freeze-drying (A) and after freeze-drying (Petri Dish of 7 cm in diameter) (B). In vitro release curve (n=6) (C).

Also, the *in vitro* release profile of formulation A1 was evaluated. Results suggested that the full drug release may be obtained in about 3 days; however, similarly to DC% outcomes, high variability was denoted, and the formulation was not considered suitable for our purpose. Still, the ability of polymer A to influence the RB release was evident and this polymer was further investigated in formulations A2 and A3.

Formulations A2 and A3 were similar. A2 consisted of an anhydrous paste made of a physical blend of polymer A, RB, and glycerol, whereas A3 consisted of a physical blend of polymer A and RB. Herein, a determined amount of the formulations was separately loaded into the implants obtaining formulations I-A2 and I-A3, respectively, which were tested for the *in vitro* release profile. However, when in contact with the acceptor medium, the formulations swelled, leading to the breakage of the permeable membrane even at the lowest amount tested (figure 6). Finally, polymer A was considered unsuitable for this investigation.

Fig. 6. Formulation I-A2 after the *in vitro* release (implants measurements: 7 x 5 mm).

3.2 Non-swellaable hydrosoluble polymer (Polymer B).

Polymer B is a not-swellaable water-soluble material that can form resistant and elastic films for several biomedical applications.

Firstly, formulation B1 was prepared through a freeze-drying technique. As in formulation A1, the final scaffold was not homogeneous in morphology (figure 7B) or drug content. The experimental DC% accounted for 0.72 ± 0.26 , revealing a coefficient of variation of 36%.

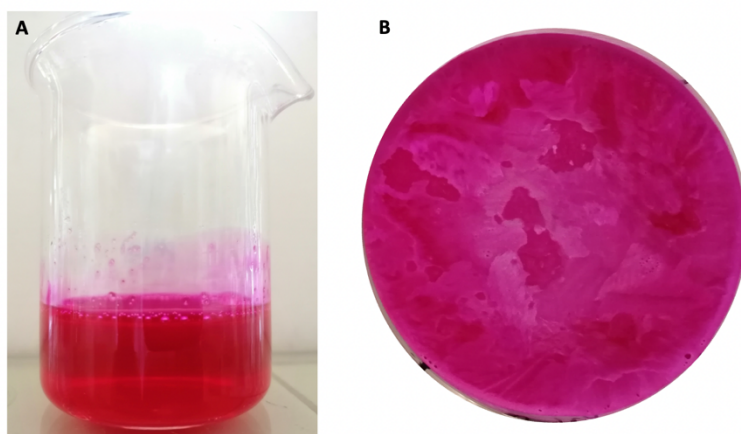


Fig. 7. Formulation B1 before (A) and after (B) the freeze-drying process.

Considering that both freeze-dried scaffolds herein formulated (A1 and B1) presented technological limitations, such as the not homogeneous DC%, it was assumed that the freeze-drying technique was not suitable for our purpose. Nevertheless, conversely to formulation A1, in the case of formulation B1 the initial aqueous solution formed was visually homogeneous (Figure 7A); thus, polymer B was further investigated.

Formulation B2 was a simple physical blend of drug and polymer B powders (figure 8C), whereas formulation B3 was represented by polymer B-based RB solid particles (figures 8E-F).

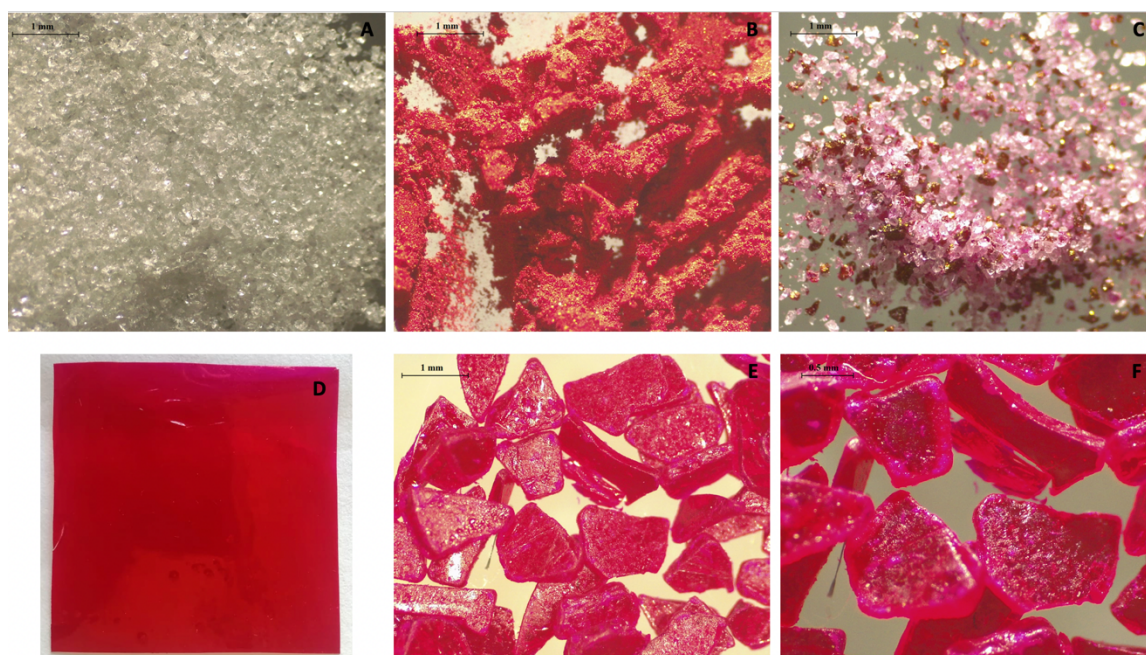


Fig. 8. Formulation B2 and B3 at optical microscopy. Polymer B powder (**A**); RB powder (**B**); formulation B2 (**C**); Anhydrous scaffold obtained by casting-solvent evaporation technique (formulation B3 before milling) (**D**); formulation B3 (**E, F**).

The optical microscopy investigation (Figure 8) evidenced the morphological differences between polymer B powder (8A) and RB powder (8B), leading to a not uniform physical mixture representing formulation B2. Conversely, formulation B3 was homogeneous in its appearance and RB content. Indeed, experimental DC% accounted for 3.68 ± 0.07 , revealing a drug loading capacity of 77.34 ± 1.53 (%) with a variation coefficient of about 2%. Furthermore, formulation B3 was tested to evaluate the *in vitro* release profile and weight loss in phosphate buffer (figure 9).

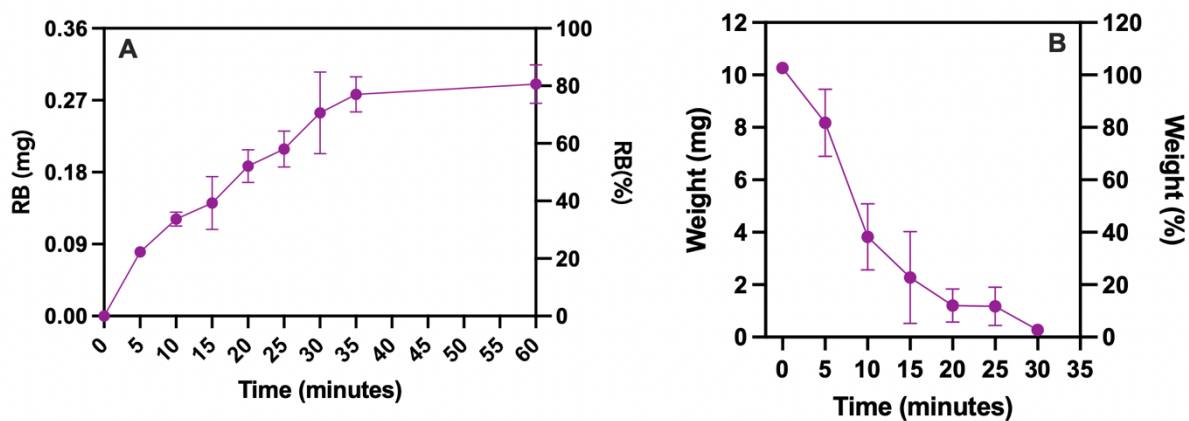


Fig. 9. *In vitro* release profile (A) and weight loss (B) in phosphate buffer of formulation B3 (n=4)

Formulation B3 provided a full RB release within 30 minutes (figure 9A), which is the time required for the complete solubilization of the particles (figure 9B).

Finally, formulations B2 and B3 were evaluated for their *in vitro* sustained release properties when loaded into the implantable device (I-B2, I-B3). Implants loaded with the pure drug (I-RB) were tested as a control. The results are shown in figure 10.

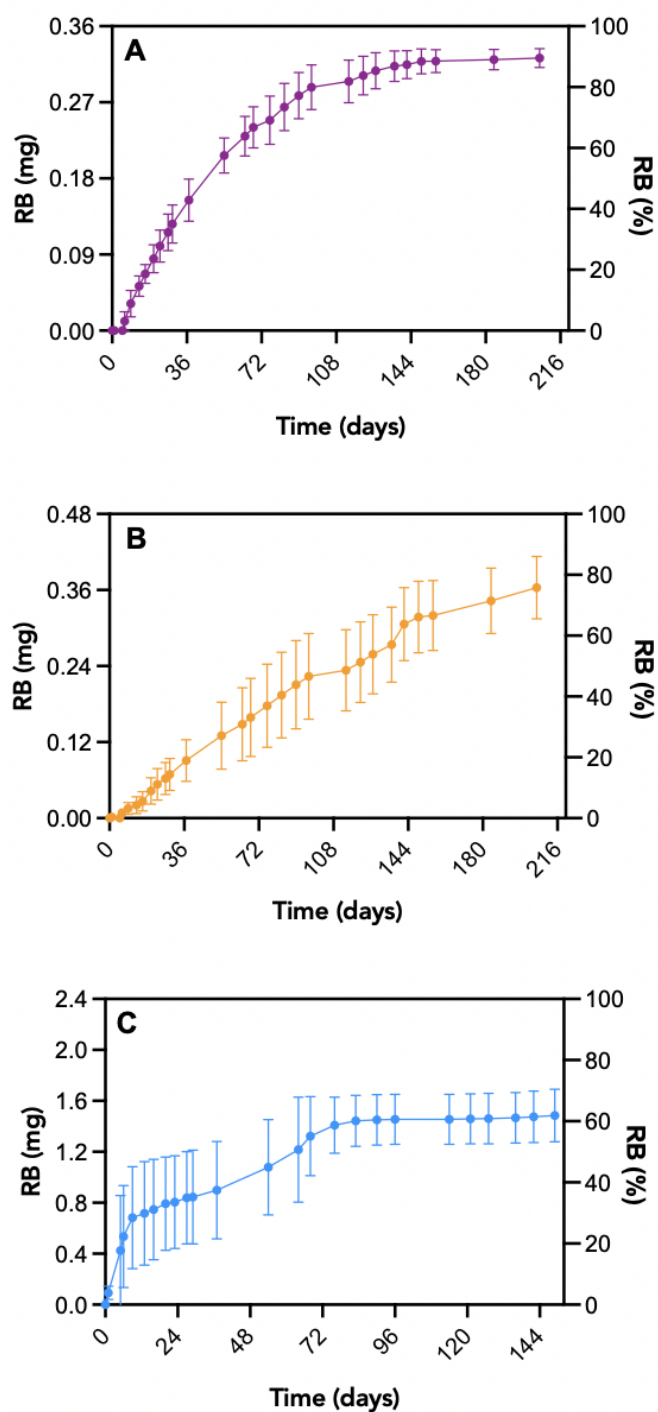


Fig. 10. *In vitro* release of I-B3 (A), I-B2 (B) and I-RB (C).

About 1.5 mg (60%) of RB was released within 75 days when I-RB was tested, and no or insignificant RB amount was released in the following weeks. A lower RB amount was tested in the I-B2 and I-B3. In the case of I-B2, the RB's release seemed to increase progressively over the period tested. However, high standard deviations were detected. The coefficient of variation of the RB amount released was higher than 20% throughout the study and was even superior to 50% in the

first time points; variability values lower than 20% were registered in the last four time points. These denote the high variability the implant I-B2 provides in releasing the drug, preventing its application in local cancer therapy, in which the precision of the spatial-temporal drug release is mandatory. This high variability is probably due to a not uniform physical blend of RB and polymer powders since the materials were still identifiable after the blending with mortar and pestle (figure 8C). Conversely, the release was much more controlled when the implant was filled with Polymer-B-based particles (formulation I-B3) without burst or dose-dumping effects. About 0.3 mg (80%) of RB was released within 96 days, and no or insignificant RB amount was released in the following weeks. The values of the coefficient of variations were lower than 20% during the first two weeks and a maximum of 10% for the rest of the study. Moreover, when the release profile of I-B3 was plotted versus the square root of time, a linear relationship was observed with the regression coefficient close to 1 ($R^2 = 0.9436$) until day 96. Conversely, an R^2 value of 0.8176 was recorded for I-B3. This data further denote the controlled released ability of I-B2 [26]. Based on this, I-B3 was selected as the leader formulation.

The final step of the current investigation evaluated the *in vitro* release of I-B4 in the agarose gel. One major drawback of the conventional *in vitro* release testing techniques is the unrealistic simulation of the conditions upon *in vivo* administration. Indeed, the environment surrounding the drug delivery systems is undoubtedly more complex than the aqueous media commonly used *in vitro* release tests. In addition, once the drug is released from the system, it must cross the tissue to reach its target site efficiently. Various phenomena are involved in this process and are not simulated when using agitated aqueous fluids as release media. To provide more realistic conditions, agarose gel has been proposed as *in vitro* release medium [27], and its application to this study is reported in figure 12.

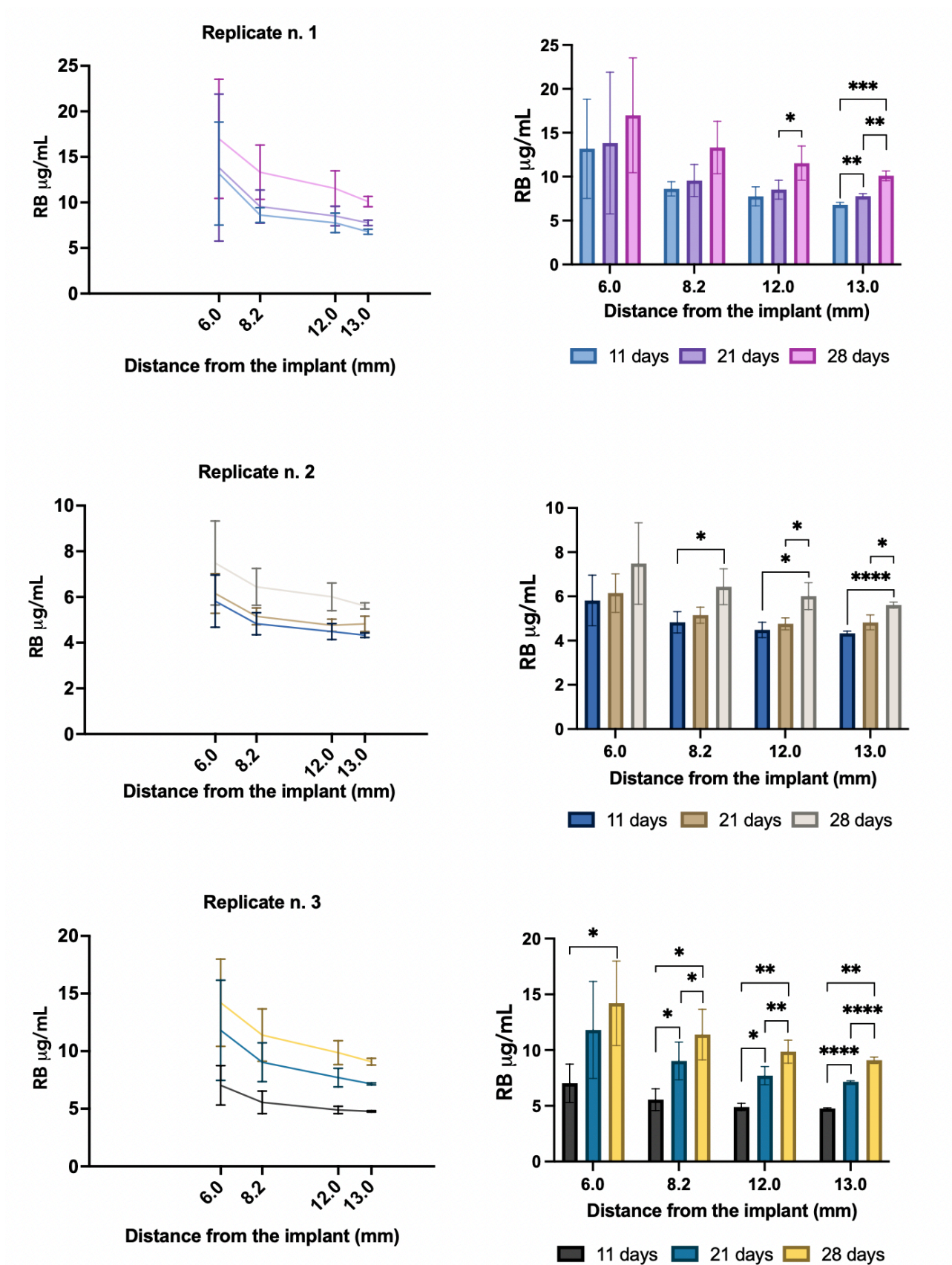


Fig. 12. Rose Bengal concentration-distance profiles within agarose gels at different time points. Results were statistically analyzed by a 2-way ANOVA test (GraphPad® Prism, version 9.4.1), and p value < 0.05 were

considered statistically significant. * p value < 0.05, ** p value < 0.01, *** p value < 0.001, **** p value < 0.0001.

The results herein reported are preliminary since the study is still ongoing. However, during the 28 days tested, I-B3 constantly released RB, which was adequately diffused through the gel (figure 13).

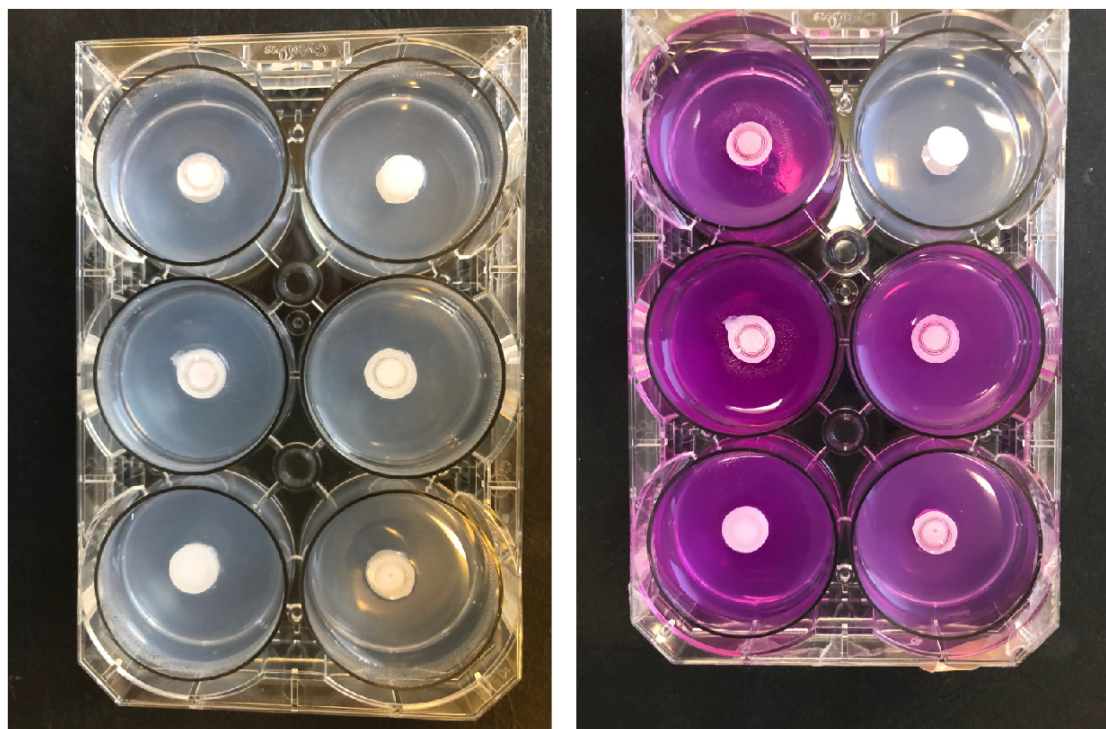


Fig. 13. Illustration of the *in vitro* release of I-B4 in agarose gel at the beginning of the study (left) and after the release (right).

4. Conclusion

To conclude, an RB long-term controlled delivery system was developed. It consists of RB loaded polymeric scaffold included in a 3D-printed PCL/PLA implantable device. The preformulation study's observations highlighted that the formulation type strongly affected the RB release profile. To better comprehend the controlled release properties of the new RB delivery system, physicochemical characterizations and *in vitro* cytotoxicity assays on cancer cell lines will be performed. Furthermore, the *in vitro* release of the device in agarose gel is ongoing. This might give a more realistic insight into the critical properties of the controlled drug delivery systems than standard bulk fluid setups.

CRedit authorship contribution statement

Sara Demartis: Conceptualization, Investigation, Methodology, Validation, Formal analysis. **Eneko Larrañeta.:** Conceptualization, Methodology, Supervision, Resources. **Anna Korelidou:** Methodology, Investigation. **Camila Julieta Picco:** Methodology, Investigation. **Ryan F. Donnelly:** Resources. **Giovanna Rasso:** Conceptualization, Methodology. **Elisabetta Gavini:** Conceptualization, Methodology, Formal analysis, Supervision, Resources.

Furthermore, for in vitro release in agarose gel: **Lise-Anne Lefol:** Investigation, Methodology. **Juergen Siepmann and Florence Siepmann:** Conceptualization, Methodology, Supervision, Formal analysis, resources.

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Conclusion

The current PhD Thesis focused on applying Rose Bengal as an anticancer agent sustained by its formulation in suitable advanced drug delivery systems.

Over the three and a half years of this project, the essential shortcoming encountered for RB formulation and drug delivery system's characterisation was represented by RB's high-water solubility and amphiphilicity. These features turn RB into a challenging molecule to formulate and deliver through biological barriers; furthermore, it was tricky to extract from most kinds of raw materials and matrices, including polymer, lipid, and bodily tissues. Indeed, despite the proven pharmacological effect and potential clinical impact, the biopharmaceutical profile of RB is the main obstacle to its affirmation in therapeutical schemes. In this regard, it's worth noting that Provectus Biopharmaceuticals', a clinical-stage biotechnology company, is developing immunotherapy medicines based on Rose Bengal. Provectus' drug platform may treat different indications across various disease areas, including oncology. However, the central administration route proposed by Provectus remains intralesional, associated with several limitations, including poor compliance and selectivity. Therefore, developing a delivery system that improves patient compliance or allows a more targeted drug localisation can further enhance the performance and application of therapeutic approaches already proposed by Provectus.

Herein, the crucial objective was to reduce the high solubility of this drug and exploit its amphiphilic tendency to obtain a proper delivery system that could maximise RB pharmacological benefits.

Regarding the cancer model, melanoma was selected among the other cancer cells sensitive to RB. This choice was mainly dependent on the highly proven efficacy of RB in eradicating this type of malignancy and on the increasingly hostile relevance of melanoma worldwide. The skin was selected as the administration route and transfersome as the primary RB carrier. The effective association of RB to the lipid carrier, based on its amphiphilicity, was highly influential in circumventing RB solubility and enhancing its cancer cell selectivity and permeation through the skin. However, this carrier was further loaded in two different microneedle array patches to move towards a more practical clinical use of RB-loaded Transfersomes. Indeed, although nanoparticles have been known for decades, particularly liposomes, their use in clinical therapy must face the significant drawback of physical stability, which can be overcome by converting them into a solid-like dosage form. Microneedle technology is slowly opening to the next era of transdermal drug delivery systems. It is believed that microneedle patches will soon be approved as therapeutics considering that their cosmetic use is already widely employed. Based on this, they were selected as the final dosage form for RB transfersomes, and the current Thesis proved their

potential. Comparing dissolving polymeric and hollow microneedle arrays, it was found that dissolving polymeric microneedles present the substantial limitation of the poor drug cargo loading that was herein represented by nanoparticles. If a local skin effect is the target, the association of nanoparticles and polymeric microneedles could be the winning strategy; still, scientific investigation is highly required to translate the application of this delivery system to systemic administration. For the last one, the “conventional” and old-standing hollow microneedles remain the most efficient and straightforward solution. As a result of these outcomes, we intend to investigate deeper the coupling of nanoparticles with microneedles to propose a biodegradable microneedle array capable of a cargo loading suitable for systemic administration.

The second investigation line, the fabrication of 3D-printed implantable devices, proposed a long-term RB delivery system that could be directly implanted close to the tumour site during surgery. In this case, no specific cancer model was selected as the aim of the study was mainly to develop a system able to promote the sustained delivery of a highly hydrosoluble molecule. The previous formulation of the drug into a polymer matrix further included in the implantable device demonstrated to be a promising technique to reach this aim. However, the current formulation strategy has the limitation that it does not enhance the lipophilic character of the drug, which is essential for improving the selectivity of cancer cells. In this regard, the current research may continue by including nanoparticles in the anhydrous polymer matrix.

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Publications and Conferences

Overall list of the Doctoral candidate's publications while attending PhD program:

1. **Demartis S**, Obinu A, Gavini E, Giunchedi P, Rassu G. *Nanotechnology-based rose Bengal: A broad- spectrum biomedical tool. Dyes and Pigments* 2021;188. Citations to 23/03/23 (Scopus): 26
2. **Demartis S**, Rassu G, Murgia S, Casula L, Giunchedi P, Gavini E. *Improving Dermal Delivery of Rose Bengal by Deformable Lipid Nanovesicles for Topical Treatment of Melanoma. Molecular Pharmaceutics* 2021;18:4046–57. Citations to 23/03/23 (Scopus): 10
3. **Demartis S**, Anjani QK, Volpe-Zanutto F, Paredes AJ, Jahan SA, Vora LK, et al. *Trilayer dissolving polymeric microneedle array loading Rose Bengal transfersomes as a novel adjuvant in early-stage cutaneous melanoma management. International Journal of Pharmaceutics* 2022;627. Citations to 23/03/23 (Scopus): 6
4. Anjani QK*, **Demartis S***, Volpe-Zanutto F, Li H, Sabri AHB, Gavini E, et al. *Fluorescence-Coupled Techniques for Determining Rose Bengal in Dermatological Formulations and Their Application to Ex Vivo Skin Deposition Studies. Pharmaceutics* 2023;15:408.
* These authors contributed equally to this work and share the first authorship. Citations to 23/03/23 (Scopus): 0
5. Slivinschi B, Manai F, Martinelli C, Carriero F, D'Amato C, Massarotti M [...], **Demartis S**, Gavini E, Comincini S. *Enhanced Delivery of Rose Bengal by Amino Acids Starvation and Exosomes Inhibition in Human Astrocytoma Cells to Potentiate Anticancer Photodynamic Therapy Effects. Cells.* 2022; 11(16):2502. Citations to 23/03/23 (Scopus): 2

Overall list of the Doctoral candidate's conference presentations while attending PhD program:

1. **S. Demartis**, G. Rassu, A. Korelidou, E. Larrañeta, R.F. Donnelly, E. Gavini. *3D-printed reservoir-type implant for the sustained release of Rose bengal in localised cancer therapy*. 4th European Conference on Pharmaceutics. Marseille, 20-21/03/2023. Poster presentation.
2. **S. Demartis**, L.A. Lefol, E. Gavini, G. Rassu, A. Korelidou, E. Larrañeta, R.F. Donnelly, F. Siepmann, J. Siepmann. *Rose Bengal loaded 3D-printed Implants: drug release into agarose gel*. 4th European Conference on Pharmaceutics. Marseille, 20-21/03/2023. Poster presentation.
3. **S. Demartis**, G. Rassu, A. Korelidou, E. Larrañeta, E. Gavini. *Reservoir-type 3D printed implantable devices to sustain the long-term release of rose bengal in localised cancer therapy*. Next Generation Chemists 2022. Cagliari, 4/11/2022. Oral presentation.
4. **S. Demartis**, Q. K. Anjani, F. Volpe-Zanutto, A. J. Paredes, S. A. Jahan, R. F. Donnelly, E. Gavini. *Dissolving polymeric microneedles as the new frontier for the targeted intradermal delivery of transfersomes: a proof of concept focused on melanoma therapy*. XXI Scuola Dottorale in Tecnologia Farmaceutica. Rome, 5-7/09/22. Oral presentation.
5. **S. Demartis**, A. Korelidou, G. Rassu, E. Larrañeta, E. Gavini. *Rose bengal loaded 3D printed biodegradable implantable devices for localized therapy of breast cancer*. Simposio Associazione Farmaceutici Industriali. Rimini, 8-10/06/2022. Poster Presentation.
6. **S. Demartis**, F. Volpe-Zanutto, A.J. Paredes, S.A. Jahan, E. Gavini, R.F. Donnelly. *Fast dissolving trilayered polymeric microneedle patch for maximising rose bengal-loaded transfersomes dermal delivery*. 8th Galenus International Workshop Valencia "The shape of things to come - Drug delivery systems from head to toe". Valencia, 27-29/04/2022. Flash oral presentation.
7. **S. Demartis**, F. Volpe-Zanutto, A.J. Paredes, L.K. Vora, S.A. Jahan, G. Rassu, E. Gavini, R.F. Donnelly. *Dissolving Microneedle Array for the controlled dermal release of Rose Bengal Loaded Transfersomes*. 13th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology. Rotterdam, The Netherlands, 28-31/03/2022. Poster Presentation.
8. **S. Demartis**, A. Korelidou, E. Magill, E. Gavini, R.F. Donnelly, E. Larrañeta Landa. *Rose Bengal loaded 3D printed biodegradable implant for long-acting in situ cancer therapy*. Autumn Meeting for Young Chemists in Biomedical Sciences 2021. 3-5/11/2021. Virtual oral presentation.

9. **S. Demartis**, AJ. Paredes, F. Volpe-Zanutto, LK. Vora, E. Gavini, RF. Donnelly. *Deformable liposomes loaded into dissolving microneedle array for metastatic melanoma targeting: a preformulation study*. NanoMedicine International Conference and Exhibition 2021. 20-22/10/2021. Virtual oral presentation.

10. **S. Demartis**, AJ. Paredes, F. Volpe-Zanutto, LK. Vora, E. Gavini, RF. Donnelly. *Nanotransfersomes- encapsulated dissolving microneedle arrays for dermal and transdermal delivery of drugs: a preformulation study*. 20th Advanced Course in Pharmaceutical Technology "Biologics in Therapy". 27-29/09/2021. Virtual oral presentation.

11. **S. Demartis**, A. Obinu, G. Rassu, P. Giunchedi, E. Gavini. *Rose Bengal-loaded Transfersomes: in vitro permeation study and stability assessment*. Controlled Release Society 2021 Virtual Annual Meeting. 25-29/07/2021. Virtual poster presentation.

12. **S. Demartis**, A. Obinu, G. Rassu, S. Murgia, L. Casula, E. Gavini. *Application of Rose Bengal in the absence of light: targeting and treating melanoma via dermal delivered Rose Bengal-transfersomes*. Nanotech-Biotech France 2021. 23-25/06/2021. Virtual oral presentation.

13. **S. Demartis**, A. Obinu, G. Rassu, S. Murgia, L. Casula, E. Gavini. *Rose-Bengal loaded transfersomes for melanoma treatment: in-vitro and ex-vivo evaluation*. 12th World Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology. 11-14/05/2021. Virtual poster presentation.

14. **S. Demartis**, A. Obinu, G. Rassu, P. Giunchedi, E. Gavini. *Lipid-Based Nanocarriers For Rose Bengal Dermal Delivery: A Promising Approach In Melanoma Treatment*. International Electronic Conference on Pharmaceutics. 1-15/12/2020. Virtual poster presentation.

15. **S. Demartis**, A. Obinu, G. Rassu, P. Giunchedi, E. Gavini. *Preparation and Preliminary Characterization of Transfersomes for Rose Bengal Topical Delivery*. Autumn Meeting for Young Chemists in Biomedical Sciences- 2020. 13-14/10/2020. Virtual oral presentation.