

Assessment of the distribution of radiolabelled non-ionic multilamellar surfactant microvesicles following topical application to canine skin biopsies: a preliminary study

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Non-ionic multilamellar surfactant vesicles (Spherulites®) represent a new generation of active ingredient carriers used for the development of topical products. This study aimed to assess the distribution of non-ionic Spherulites® into canine skin. A suspension of Spherulites® labelled with encapsulated 14C-palmitic acid was applied to the epidermis of cutaneous biopsies. Control biopsies were treated with non-encapsulated probe or left untreated. The distribution of radioactivity was assessed in cryostat sections by autoradiography and by direct radioactive imaging. Examination of sections showed that signals from the non-encapsulated probe were present at the skin surface. The distribution of radioactivity from the encapsulated probe (in non-ionic Spherulites®) showed signals in the living epidermis, in hair follicles and in the dermis. This study suggests that non-ionic Spherulites® are able to penetrate deeply into the skin of the dog.

Key words: Spherulites – Dog – Skin – Penetration – Radio imaging – Topical therapy.

Spherulites® are multilamellar microvesicles composed of surfactants, which represent a new generation of systems for the encapsulation of active ingredients [1, 2]. They allow control of the release of active substances applied to the skin [3]. The ability of cationic Spherulites (positively charged) to bind to the negatively charged hair surface has already been demonstrated by scanning electron microscopy showing Spherulites® on the surface of treated hair [4]. These properties, allowing progressive and controlled release of active substances at the skin surface, are now being used in the development of veterinary dermatological products (Allerderm®, Virbac). When deep sites need to be reached, attachment to the skin surface is not required and non-ionic Spherulites (neutral microvesicles) may represent appropriate vectors owing to their ability to diffuse through cutaneous structures. The aim of this experimental study was to assess the distribution of non-ionic Spherulites® in canine skin after a single topical application.

I. MATERIALS AND METHODS

1. Biopsy sampling and procedure

Eight cutaneous biopsy specimens were used for this study. Biopsy sampling was performed in two series of experiments (separated by one month) on a single dog and in the same skin area (flanks) to limit interanimal variation. Skin biopsies were performed by a veterinary surgeon, with a 6-mm biopsy punch, on a healthy dog under general anaesthesia with ketamine. The specimens were immediately placed on moist paper and transferred to plastic vials held on ice; they were used for the study within 2 h of sampling.

2. Biopsy treatments and autoradiography procedure

The specimens were placed on a glass surface with the epidermis facing upwards. A suspension of non-ionic Spherulites® encapsulating 14C-palmitic acid (1.66 MBq/ml) was dropped onto the epidermal surface of 5 biopsy specimens held for two contact times, 30 min (n = 3) and 60 min (n = 2). Application of the Spherulites® suspension (dilution 20% in deionised water) was performed as follows: 50 µl was dropped on each biopsy with a micrometer syringe avoiding contamination of the side or base of the specimen, in open air, at room temperature (20°C) and humidity without additional drying. Three control biopsies were treated as follows: one received non-encapsulated 14C-palmitic acid (0.02% in mass with 98.91% of deionised water, 0.31% of a polymer, Rhodicare, Rhône Poulenc, and 0.76% of a surface active substance, Montanide 103, Seppic) with a contact time of 30 min (n = 1); one was treated for 30 min with non-labelled Spherulites® and one received no treatment (Table I). All biopsies were then rinsed separately by immersion in sterile water for 1 min and left in the air at room temperature to dry for 30 min. The biopsies were frozen, by dipping for 20 s into isopentane cooled in liquid nitrogen, after which they were placed in vials and held in liquid nitrogen for 5 min. The biopsies were then transferred to a chamber at -20°C for 2 h before being cut. Biopsy sections were performed with a cryostat. The thickness of each section was 14 µm. Twenty sections from each biopsy were placed on glass slides. The slides were then dipped in a photographic emulsion (LM1; Amersham) and kept in a dry, dark box for 15 days before being developed and stained with



Table I - Summary of the different treatments and exposure times.

Exposure time (min)	Treated biopsies: 14C-palmitic acid in Spherulites®	Treated biopsy: 14C-palmitic acid non-encapsulated	Control biopsy: unlabelled Spherulites®	Control biopsy: no treatment
30	3	1	1	
60	2	-	-	
total	5	1	1	1

Table II - Silver grain counting in anatomical structures.

Anatomical region	Epidermis		Hair follicles		Dermis		Hypodermis	
	Invest. 1	Invest. 2	Invest. 1	Invest. 2	Invest. 1	Invest. 2	Invest. 1	Invest. 2
Biopsies treated with 14C-palmitic acid in Spherulites®								
Contact time 30 min	4	4	3	2	2	2	1	1
Contact time 60 min	4	4	2	2	3	2	1	1
Biopsy treated with non-encapsulated 14C-palmitic acid	4	4	1	1	1	1	1	1
Control: biopsy treated with unlabelled Spherulites®	1	1	1	1	1	1	1	1
Control: no treatment	1	1	1	1	1	1	1	1

Average results obtained by investigator 1 and investigator 2 (20 sections examined per biopsy). Presence of silver grains estimated in a 1 to 4 level scale: 1, none (= background), 2, low (up to 2 x the background), 3, medium (up to 5 x the background), 4, high (more than 5 x the background).

haematoxylin and eosin. Twenty coded photomicrographs were then examined blindly by two investigators. For this purpose the photomicrographs were divided into numbered sectors representing anatomical regions i.e. epidermis, hair follicles, dermis and hypodermis. In each sector, the presence or absence of silver grains was estimated on a 1 to 4 level scale (Table II), independently by each investigator.

3. Quantitative detection of 14C-beta particles

At the same time, direct measurement of radioactivity was made in a total of 12 additional sections, 6 from biopsies treated with the encapsulated probe and 6 from biopsies treated with the non-encapsulated probe. This was done using a high resolution radio imaging system (micro-imager; Biospace-Mesures, Paris, France) [6] in which quantitative detection of 14C-beta particles is made by means of a solid scintillator sheet coupled to an image-intensifying tube and an electronic camera. This system enables display of the spatial distribution of radioactivity in the samples and allows the video image of the tissue to be superimposed (on the radioactive profile).

II. RESULTS AND DISCUSSION

Autoradiography showed the presence of a large number of silver grains in the anatomical structures. Assessment by the two investigators gave identical results (Table II).

In sections from biopsies treated with the encapsulated probe (in Spherulites®), they were mainly present in the epidermal cell layers but also within hair follicles and follicular infundibula, and in the dermis (Figure 1 - A).

In biopsies treated with the non-encapsulated probe, the silver grains were abundant in the superficial cellular layers of the epidermis. Very little radioactivity was detected in hair follicles (Figure 1 - B). In both instances, no radioactivity was detected or measured in the hypodermis and, qualitatively, no difference was observed between 30 and 60 min contact time (Table II).

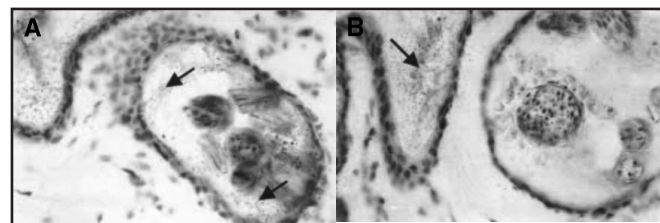


Figure 1 - A: Autoradiography of a frozen section treated with the encapsulated probe (in Spherulites®) showing the presence of silver grains in the epidermal cell layers and in the hair follicle infundibula (→). Magnification x 500. B: Accumulation of silver grains (→) on the epidermal surface with the non-encapsulated probe. Very few grains are present at the level of the hair follicle. Magnification x 500.

Direct quantification of radioactivity by micro-imaging confirmed that the signal with the non-encapsulated probe was distributed quite evenly at the skin surface (Figure 2, profile a). The radioactivity with the encapsulated probe showed a patchy distribution at the skin surface but there was an increased signal in the deeper parts of the biopsy section (Figure 2, profile b), consistent with the penetration of non-ionic Spherulites® into the cutaneous structures (including the hair follicles and the dermis).

No radioactivity was detected in the frozen biopsy treated with unlabelled Spherulites® and in the untreated frozen biopsy.

The differences observed in the location of radioactive signals between the sections treated with the encapsulated probe (in Spherulites®) and those treated with the non-encapsulated probe suggest that non-ionic Spherulites® promote the diffusion of the radioactive probe into the skin. Furthermore, the abundance of radioactive signals along and within the hair shafts suggests that non-ionic Spherulites® may also follow the route of the hair follicle to penetrate into the deep cutaneous structures (including the deep dermis layers). This complies with previous observations made by Betz *et al.* where fluorescence labelled phospholipids penetrated along the hair shafts into deeper layers of the human dermis, *in vitro* [7].



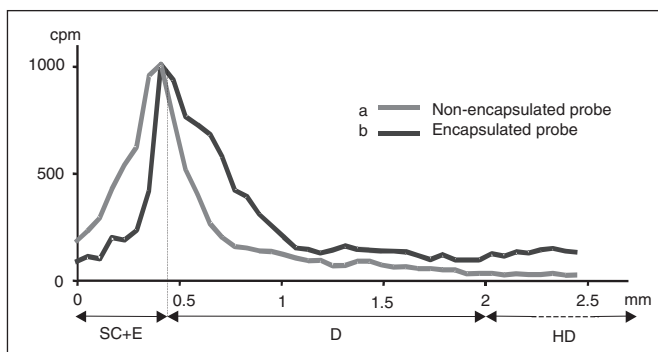


Figure 2 - Normalised radioactivity profiles following direct quantification of radioactivity by micro-imaging. In 2a, the signal from the non-encapsulated probe is detected with a maximum at the skin surface, mostly in the stratum corneum and epidermis (SC + E), but not in the dermis (D) and hypodermis (HD). In 2b, the encapsulated probe is detected principally in the deeper parts of the section (hair follicles and dermis).

These observations support the concept that non-ionic Spherulites® diffuse through epidermal cell layers as well as into cutaneous appendages (hair follicles) and into the dermis.

The findings confirm the usefulness of non-ionic Spherulites® for the delivery of active substances into the skin. This encapsulating system may therefore be of value in the topical treatment of skin diseases in dogs, especially when deeper sites must be reached.

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