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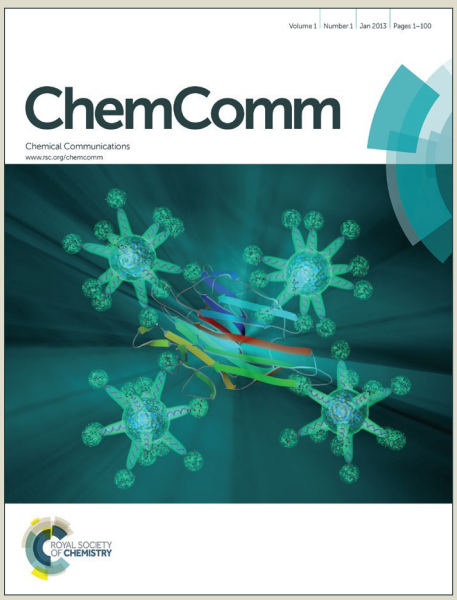

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ARTICLE TYPE

An easy-to-detect nona-arginine peptide for epidermal targeting

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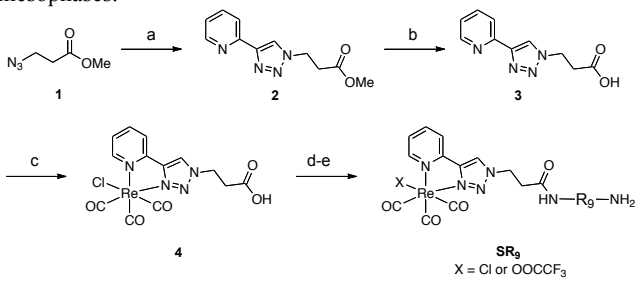
A correlative approach combining synchrotron radiation based IR microscopy and fluorescence microscopy enabled the successful detection and quantification of a nona-arginine peptide labelled with a Single Core Multimodal Probe for Imaging (SCoMPI) in skin biopsies. The topical penetration of the conjugate appeared to be time dependant and occurred most probably *via* the extracellular matrix.

Vibrational excitation in the infrared (IR) is attractive for chemical-imaging[1] as it does not induce photo-bleaching, contrary to fluorescent imaging based on electronic transitions.[2, 3] Moreover IR spectroscopy can be used to probe local environment without the need for labels or staining, relying on the specific vibrational absorption of biomolecules. However fluorescence is more spatially resolved (<100 nm whereas the resolution is over the µm in the mid-IR in the case of an optical detection limited by diffraction and the Abbe criteria). Thus probes detectable by several techniques are of interest and their use opens up the opportunity for cross-correlative studies.[3] Combining both modalities in a single molecular unit is a challenge we have raised recently. We showed that metal-carbonyls (M-CO) can be mapped inside cells using their IR-signature *i.e.* an intense absorptions in the mid-IR transparency window of biological media (in the 2000 cm^{-1} range).[4, 5] Rhenium tris-carbonyl complexes *fac*-Re(N^N)$(CO)_3$ with appropriate ancillary N^N are luminescent and this property has been often used in subcellular bio-imaging.[6-14] We envisioned that such compounds based on a single M-CO core could be used as bimodal IR and luminescent probes, that we named **SCoMPI**s for **Single Core Multimodal Probe for Imaging**.[2] We demonstrated that correlative cellular imaging can be achieved using a SCoMPI[2, 15] and a recent study has focused on the relationship between the lipophilicity, the cellular uptake and the cytotoxicity of SCoMPI derivatives.[16] Combining IR and fluorescence microscopies is interesting since it enables working at different scales, which is usually highly challenging with a single molecule. Indeed, with fluorescence microscopy sub-cellular resolutions are routinely achievable, however, background emission and low tissue penetration often hamper tissue imaging. On the contrary, IR resolution is limited to a few micrometers but the deeper tissue penetration of IR radiation makes IR imaging a technique of choice for tissue imaging.

In order to take advantage of these complementary properties we have conjugated a SCoMPI with a nona-arginine peptide to monitor the transdermal penetration of the conjugate through human skin biopsies. Application of peptides for transdermal delivery has recently aroused attention in cosmetics and pharmaceutical fields.[17, 18] A pioneer work by Rothbard *et al.* showed that the conjugation of cyclosporin A with an hepta-arginine peptide enabled the delivery of the cyclosporin A not only into the dermis but also into tissue T cells, whereas the unconjugated cyclosporin A exhibits a poor topical absorption.[19] More recently, similar peptides have been successfully used for the transdermal delivery of a variety of molecules: peptides,[20] drugs,[21, 22] RNA [23] and nanoparticles (either covalently attached[22, 24] or through delivery systems involving non covalent association[20, 23, 25-27]), lipid vesicles[28] or liquid crystalline mesophases.[21]



Scheme 1 Synthesis of the SCoMPI conjugated with the nona-arginine peptide **SR**$_9$. Conditions: a) 2-ethynylpyridine, $CuSO_4$, sodium ascorbate, acetone/H_2O, r.t., 2h, 54%. b) LiOH, THF/H_2O, r.t., 2h, 50%. c) $Re(CO)_5Cl$, toluene/MeOH, reflux, 6h, 72%. d) N-hydroxysuccinimide, EDC, DIPEA, DMF, r.t., overnight, 13%. e) $^+NH_3$-R_9-$CONH_2$, DIPEA, DMF/H_2O, 33%, HPLC purification (preparative C18 column, flow: 14 mL/min, gradient: from 15% to 30% of CH_3CN in H_2O over 30 min both solvents containing 0.1% TFA).

In order to easily graft the SCoMPI onto R_9,[29] a nona-arginine peptide, a SCoMPI bearing a carboxylic acid function has been synthesized (**4**). The 4-(2-pyridyl)-1,2,3-triazole ligand (pyta) has been obtained *via* an alkyne-azide click reaction catalyzed by copper from methyl 3-azidopropionate **1** prepared according to a previously published procedure.[30] After saponification of compound **2**, the *fac* rhenium tricarbonyl complex (**4**) was formed in good yields and isolated by precipitation. The succinimidyl ester was then generated, coupled to the N-terminus of R_9 peptide in solution and purified by HPLC to obtain the **SR**$_9$ conjugate. An exchange of the chloride ligand in the Re-Cl core cannot be excluded in water, in coordinating solvents, or in biological

environments, as reported by others and us.[31, 32, 33] However, the (pyta)Re(CO)$_3$Cl core was found to be stable in DMSO up to 4 days at room temperature.[33] Because of this possible lability of the chloride-rhenium bond, it is likely that during the HPLC purification, an exchange of the chloride with a trifluoroacetate occurred, which would not modify the properties of the conjugate which is highly charged due to the peptide moiety. Aqueous **SR$_9$** solutions at 2 10^{-2} M were applied to the surface of human skin mounted in a Franz diffusion cell. After 6 or 24 hours, the skin samples were washed, frozen and 10 µm tissue sections obtained using a microtome were mounted on CaF_2 windows (see SI). The distribution of **SR$_9$** in human skin slices was first determined by synchrotron radiation FTIR spectromicroscopy (SR-FTIR-SM, see SI) by integrating IR one of the CO bands (A1) of the SCoMPI, as previously described.[4, 16] Luminescence imaging was performed on skin sections stained with DAPI. DAPI reveals the nuclei and hence stains the viable epidermis (VE) (see Fig. 1 and 2, purple staining).[34]

After a 6 h exposure, **SR$_9$** was mainly distributed in the *stratum corneum* (SC) and was not detected in the deeper layers of the epidermis (Fig. 1). After 24 h (Fig. 2), **SR$_9$** was found in the whole epidermis, from the SC to the *stratum basale* (SB). Very interestingly, no compound was detected deeper than the dermo-epidermal junction. This observation is of great interest as an active ingredient in the cosmetic field should accumulate in the non-vascularised epidermis (SC and VE), and not reached the dermis, which is connected to the systemic circulation. Both IR and luminescent spectroscopies were consistent, showing **SR$_9$** at the same location on all observed biopsies (see Fig. 1 and 2).

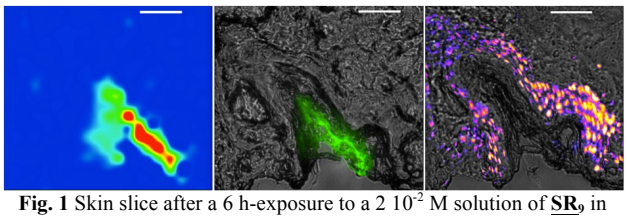


Fig. 1 Skin slice after a 6 h-exposure to a 2 10^{-2} M solution of **SR$_9$** in water. ***Left***: mapping of the integral of the absorbance of the A$_1$-band (2040-2000 cm^{-1}). ***Middle***: bright field image merged with the luminescence signal of **SR$_9$**. ***Right***: bright field image merged with the staining of nuclei by DAPI. Scale bar 40 µm.

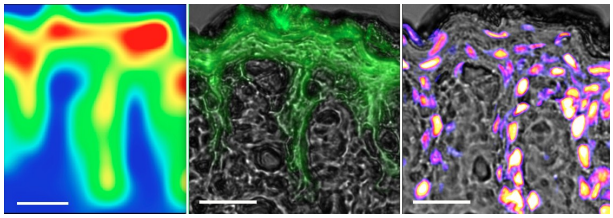


Fig. 2 Skin slice after a 24 h-exposure to a 2 10^{-2} M solution of **SR$_9$** in water. ***Left***: mapping of the integral of the absorbance of the A$_1$-band (2040-2000 cm^{-1}). ***Middle***: bright field image merged with the luminescence signal of **SR$_9$**. ***Right***: bright field image merged with the staining of nuclei by DAPI. Scale bar 20 µm.

Three mechanisms are commonly proposed for the transdermal penetration of peptides conjugates: the transcellular route, the appendage route and the intercellular route.[17] In order to get more insights into the topical penetration mechanism of **SR$_9$**, keratinocyte cells, the main cellular constituent of the human epidermis, were incubated with an aqueous solution of the conjugated peptide. HaCat cells (human keratinocyte cell line) were incubated with the **SR$_9$** (1 h, 50 µM), fixed (PFA 4%, see SI) and imaged by fluorescence microscopy. Under these conditions, no apparent cytotoxicity of **SR$_9$** was measured according to the MTT Formazan and LDH test (see SI). Surprisingly, only few cells showed a fluorescent signal with a cytoplasmic distribution of the labelled peptide (data not shown). This observation means that most of the cells do not internalize the peptide, suggesting that the intracellular route is probably not the main route for the transdermal penetration. It is more likely that the peptide diffuses through the epidermis *via* the extra-cellular matrix.

Since the quantum yield of most probes is highly dependent on the environment,[35] luminescence cannot be directly implemented for intracellular quantification.[36,16] An important and useful feature of IR spectroscopy is the possibility of easy-to-perform and reliable quantification. M(CO)$_3$ derivatives show two IR-bands in the mid-IR,[37] one is labelled A$_1$ (singly degenerated band) and the other is labelled E (doubly degenerated band) at approximately 2020 and 1915 cm^{-1}, respectively. The A$_1$-band is known to be appropriate for titration,[37,16] and we previously showed that it can be applied for a direct quantification within a collection of cells with a calibration curve.[4, 16] Using a similar procedure[16] (see SI and Figure S2 for the calibration curve), **SR$_9$** was quantified in two different areas of the skin samples, the SC and the VE, respectively (Table 1 and Table S1 for more details).

Table 1. Quantification of **SR$_9$** in two compartments of the skin slices in hotspots. A corresponds to the absorbance of the A$_1$-band (see table S1 for the details). The ROI (region of interest) corresponds to the beam section, hence is of 10x10 µm^2 on 10-µm thick (thickness of skin slices).

	After 6 h		After 24 h	
	In the SC	In the VE	In the SC	In the VE
A at 2025 cm^{-1} (a. u.)	30 ± 5	2.1 ± 0.3	5 ± 2	20 ± 2
N (mol) *per* ROI	8.2 10^{-15}	6.0 10^{-16}	1.4 10^{-15}	5.4 10^{-15}

After 6h, the accumulation is clearly non-homogenous in the skin, with spots where **SR$_9$** accumulates (hotspots) and large areas showing no product (see Fig. 1). We have chosen hotspots in several sections (see Table S1) and we have estimated in these regions the relative proportion found in the SC and in the VE, the VE being revealed by DAPI staining (see above). Values from Table 1 are given for a region of interest (ROI) corresponding to a surface of 10x10 µm^2 (beam size) and 10-µm thick skin slices.

After 24 h, the quantity of **SR$_9$** in the VE is in the same range as the quantity in the SC. Hence qualitative images were confirmed through the IR quantitative analyses at different skin depths: **SR$_9$** can be accumulated in the VE, up to the dermo-epidermal junction.

In summary, we labelled a nona-arginine peptide with a SCoMPI in order to image the conjugate by correlative IR and luminescence microscopies. Combining these imaging techniques enabled to work from the cell to the tissue scales and also to quantify the conjugate on the skin biopsies. At a short exposure time (6 h), the conjugate stayed mainly in the SC whereas it was

also detected in the deeper layers of the epidermis for a long exposure time (24 h). Interestingly, the SCoMPI-peptide conjugate was not found in the dermis. This is of great interest in the cosmetics field since the conjugate could serve as a vector of a biologically active ingredient for the targeting of the epidermis constituents while preventing its diffusion through the blood circulation.

Abbreviations

VE: Viable Epidermis; SC: Stratum Corneum; SR-FTIR-SM: synchrotron radiation FTIR spectromicroscopy; SCoMPI: Single Core Multimodal Probe for Imaging.

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Notes and references

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† Electronic Supplementary Information (ESI) available: (1) Synthesis and characterization of **SR$_9$** ; (2) Skin preparation (3) Synchrotron radiation FTIR spectromicroscopy (SR-FTIR-SM) (4) HaCat cells culture and luminescence microscopy (5) FTIR quantification (6) Biological tests. See DOI: 10.1039/b000000x/

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