Bioconjugate Chemistry

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Communication

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Labeling of hyaluronic acids with a Re-tricarbonyl tag and percutaneous penetration studied by multimodal imaging

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Hyaluronic acids were labeled with a Re-tricarbonyl used as Single Core Multimodal Probe for Imaging and their penetration into human skin biopsies was studied using IR microscopy and fluorescence imaging. The penetration was shown to be dependent on the molecular weight of the molecule and limited to the upper layer of the skin

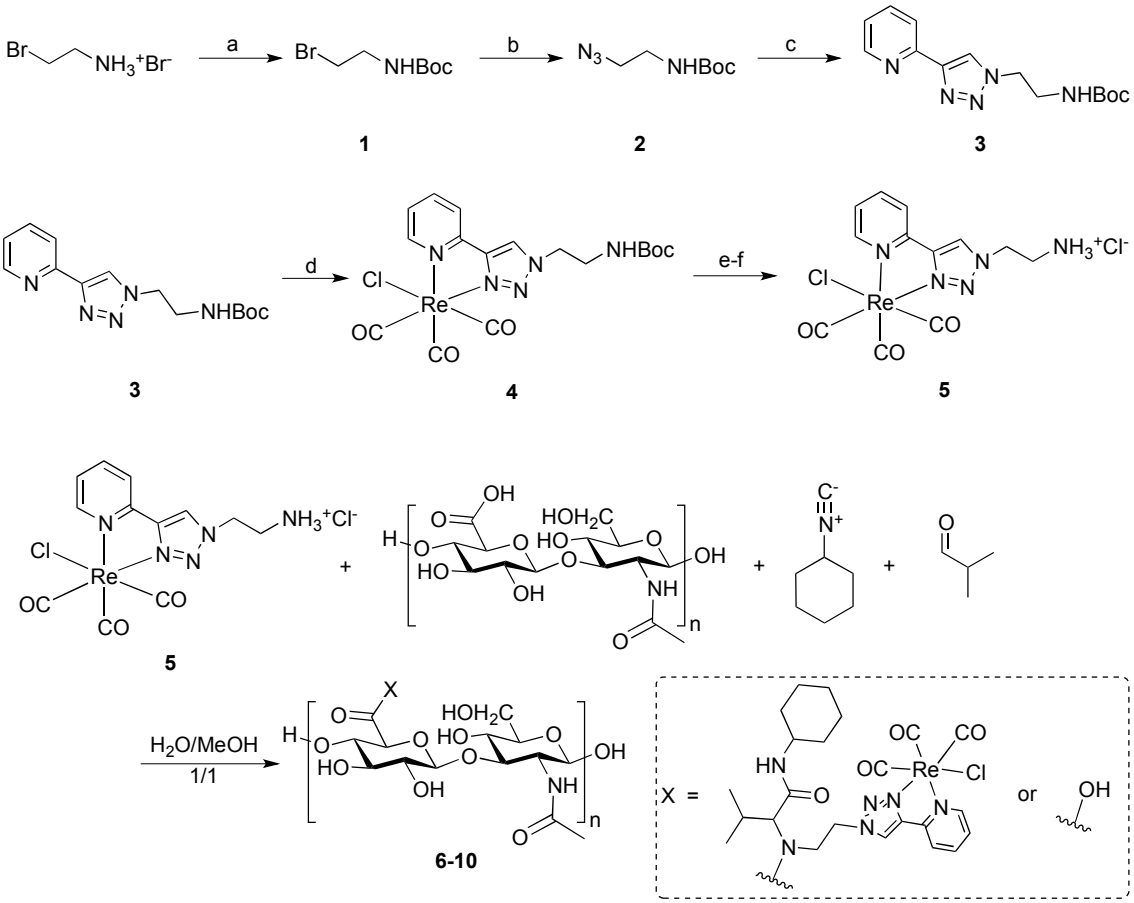
Hyaluronic acid (HA) is a polymer of disaccharides made of D-glucuronic acid and D-*N*-acetylglucosamine moieties, linked by alternating ß-1,4 and ß-1,3-glycosidic bonds,[1] with molecular weights (MW) up to 10^7 Da. HA is found in tissues and body fluids of almost all living organisms.[2] This highly hydrophilic molecule binds a thousand times its volume of water,[3] and is commonly used as a medicine, for instance injected into joints to treat arthritis or in dermis to fill facial wrinkles.[4] Low MW HAs are only used to maintain hydration of epidermis after topical application whereas high MW HAs are also used as filing agent after cutaneous injection.[3]

In order to investigate their penetration into skin, HAs of two MW have been labeled with a multimodal Re-tricarbonyl probe. Several $Re(CO)_3$ tags bearing a fluorescent and an IR modality on the same molecular moiety have been recently developed. These SCoMPIs —for Single Core Multimodal Probes for Imaging—[5-7] show two characteristic IR-absorption bands in the transparency windows of biological tissues in the mid IR (between 1800 and 2200 cm^{-1}), namely E and A_1 for the antisymmetric and symmetric CO-stretching vibrations.[7-9] They have already been characterized and used as organelle trackers[5, 10-12] or for the labeling and imaging of molecules of biological interest,[6, 7, 13-16] such as amino-acids and peptides,[11, 15-20] peptide-nucleic acids,[21] vitamins,[22] hormones,[23] or medicines.[24] Interestingly, the A_1-band, that is a singly degenerated band, can be used for quantification purpose.[9, 25-28] IR-imaging is a challenging technique. Its lateral resolution is limited to a few µm ($\lambda/2$, so about 2.5 µm at 2000 cm^{-1} or 5 µm) when the detection is optical but resolution down to 20-50 nm can be obtained using near-field techniques, such as AFM-IR.[29, 30] IR-imaging can be recorded on dedicated synchrotron beamlines (synchrotron-based FTIR spectromicroscopy or SR-FTIR-

SM) but also on IR-microscopes that are commercially available with focal-plane array detectors enabling fast imaging and this will certainly popularize FTIR-based spectromicroscopy (FTIR-SM) in biology. One interest of the SCoMPIs as IR-probes is their reliability for quantification in biological environments in which polarities have a wide range of diversity. Generally speaking, luminescence cannot be directly implemented for absolute quantification, since the quantum yield is highly dependent on the environment.[6, 31, 32] In contrast, the epsilon of the A_1-band of the $Re(CO)_3$ moiety is not strongly dependent on the solvent.[6, 7, 28] Therefore, this band can be used for absolute titration, as previously performed.[9, 18, 26-28] These SCoMPIs $Re(CO)_3$ probes are thus of interest, both for IR-mapping and quantification purposes, and their grafting onto biomolecules worthy to explore.

To graft the probe onto the HA, a Ugi multicomponent condensation involving the carboxylic acid functions of HA[33] was selected for its high efficiency in water,[34, 35] the most appropriate solvent for HA.[35] A graftable SCoMPI bearing an amine moiety was synthesized in five steps and was reacted with HA, as described in Scheme 1 and SI.

HAs in two ranges of MW were used (10 kDa and 400-1000 kDa), with a low or high probe to HA (probe/HA) ratio (Table 1). In order to easily monitor the reaction completion using ^{1}H-NMR, the Ugi reaction was performed in deuterated solvent (D_2O/CD_3OD 1/1). New signals growing in the aromatic range (7.5-9.5 ppm) were assigned to the probe grafted on HA (Figure S3, SI). When no more evolution could be seen, the tagged HAs were purified by filtration over a membrane with a 3000 MW cut-off, in order to remove low-molecular weight reactants, and the fraction containing HA was then lyophilized.



Scheme 1. Synthesis of the graftable SCoMPI **5** and labeling of HA using a Ugi condensation. (a) Boc_2O, DIPEA, THF, 1h, r.t., quantitative. (b) NaN_3, NaI, acetone/H_2O, 40h, 35°C, 66%. (c) 2-ethynylpyridine, $CuSO_4$, sodium ascorbate, acetone/H_2O, 3h, r.t, 62%. (d) $Re(CO)_5Cl$, toluene, 1h30, 80°C, 88%. (e) CH_2Cl_2, TFA, 1h, 25°C. (f) CH_3OH, HCl, 10 min, 25°C, 95%.

IR-spectroscopy was used to characterize the tagging of the HAs. The number of bands of CO-stretching of M(CO)s observed in the 2000 cm^{-1} wavenumber range depends on the local symmetry, with the doubly degenerated band labeled E possibly split in the case of a low symmetry.[8] Their energy position vary with the local environment, depending on the hydrophobicity of the environment for instance, and splittings can be observed upon aggregation or in the solid state when there is a composite environment.[8, 9] The IR-spectra of **4** and **5** have been recorded in several solvents (Figure S4 and table S2, SI). They show the two bands in the 2000 cm^{-1} energy range. As previously published in the case of pyridyl-triazolyle based $Re(CO)_3$, the E-band is split into a doublet due to a low symmetry.[5-7, 28] As shown in figure S4 and table S2, the positions of the A_1 band and E bands are shifted depending on the solvent nature, with shifts of about 10 to 20 cm^{-1}. Similar shifts have already been reported, with frequencies higher in hydrophobic than in hydrophilic environment.[36] The spectra of the tagged HAs show several sets of A_1 and E bands, as exemplified with **7** in figure 1. The presence of several sets of A_1 and E bands is indicative of the different local environments of the $Re(CO)_3$ moiety which can be found in a hydrophobic or hydrophilic environment depending on the fact that the SCoMPI is exposed to the solvent or buried within the polymer, where its environment may be composite and solid-state like.

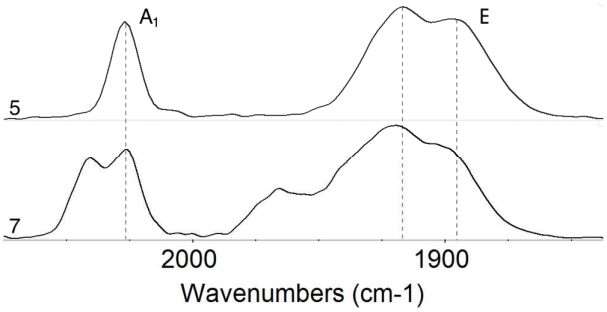


Figure 1. IR spectra obtained using ATR mode between 1820 and 2075 cm^{-1} of **5** in acetone and **7** in water (2 $mg \cdot mL^{-1}$). See also Figure S4 and Table S2 for the spectra of **5** in a range of solvents.

The number of grafted probes *per* monomeric unit (one D-glucuronic acid and one D-*N*-acetylglucosamine moiety) was evaluated by ^{1}H-NMR when possible (compounds soluble enough: **6** and **7**) and FTIR using a calibration curve with a mixture of HA and **5** (SI). The results obtained by FTIR for the lightest compounds **6** and **7** were in good agreement with those obtained by ^{1}H-NMR. This validates the FTIR protocol that was then applied for the heavier compounds (**8**, **9**, **10**) for which NMR quantification could not be used (see Table 1).
To investigate their ability to permeate through skin, 200 µL of a 2 $mg \cdot mL^{-1}$ aqueous solution of labeled HAs was applied to the surface of human skin biopsies mounted in a Franz cell chamber. After a 7- or 24-hour exposure, skin samples were washed, frozen and cut in 10-µm thick sections that were mounted on CaF_2 windows.

The distribution of the labeled HAs in the skin was investigated using synchrotron radiation FTIR spectromicroscopy (SR-FTIR-SM, see Figure 2) or FTIR spectromicroscopy (FTIR-SM, see SI, figures S8-S10). IR spectra were recorded in the 800-4000 cm^{-1} range, and maps were generated by integrating specific bands (see SI): A_1 band (2055-2005 cm^{-1}) and CH_2 bands in the 2868-2838 cm^{-1} range. Areas with high concentration of CH_2 indicate a lipid-rich layer, corresponding to the *stratum corneum* (SC).[18, 37] Luminescence imaging was also performed after Hoechst staining[38] to reveal cells nuclei of the viable epidermis (VE).[18]

Table 1. Distribution of HAs in the skin

Molecule	MW of HA (kDa)	Labeling % (IR)[a]	Labeling % (NMR)[b]	Soluble in water	Distribution in the skin	
					7h	24h
6	10	~5%	~3-4%	Yes	Not observed	Hotspots mapped by IR and fluorescence in the SC (Figure S8)
7	10	~20%	~20%	Yes	Hardly detectable by IR, hotspots mapped by fluorescence in the SC (Figure S9)	Homogeneously distributed in the SC and very weak amount in the VE (Figure 2, IR and fluorescence)
8	10	~40%	-	No	/	/
9	400-1000	~20%	-	No	/	/
10	400-1000	~5%	-	Yes	Not observed	Inhomogeneous, hotspots in the SC (Figure S10)

[a] % of functionalized carboxylic acid functions determined by IR. [b] % of functionalized carboxylic acid functions determined by ^{1}H NMR.

After a 24-hour exposure, **6** was hardly detectable by infrared imaging but was detected by fluorescence microscopy at some spots where it accumulated (Figure S8, SI). The labeling

ratio of **6** was probably too low for an IR-detection. Compound **7**, with a labeling rate c.a. four times higher, was thus investigated: its distribution after a 7-hour exposure was non-homogenous in the skin section, with spots of high accumulation (or hotspots) and large areas showing no product (Figure S9, SI). After 24 hours, an intense and homogenous signal was observed both by IR and fluorescence and it was mainly observed in the SC. Only some weak signals were seen at few areas in the VE (Figure 2). The high MW analog with 5% tagging (**10**) was investigated and showed an inhomogeneous distribution at 24 h. Compounds **8** and **9** were not soluble enough for an epidermal application. The results are summarized in Table 1.

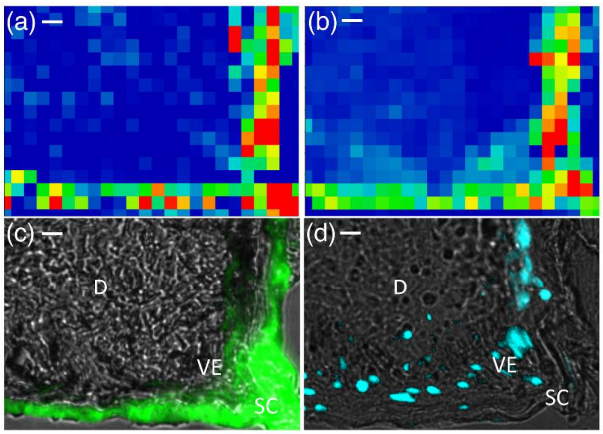


Figure 2. Map of a 10 µm-thick skin cryosection after a 24-hour exposure with **7**, mounted on a CaF_2 window (scale bar = 10 µm) (a-b) SR-FTIR-SM images based on the integration of absorption bands: (a) A_1-band (2055-2005 cm^{-1}), (b) CH_2 (2868-2838 cm^{-1}), using a false color scale from blue (low) to red (high intensity), (c, d) Bright field image merged with (c) the luminescence signal of **7** (ex 350/50x, em 560/80m) and (d) nuclei staining (Hoechst, blue, ex 350/50x em 460/50m); 128 scans, 8 cm^{-1} spectral width. D=dermis, VE=viable epidermis, SC=stratum corneum.

FTIR-SM was also used to investigate modifications of lipids and proteins of the skin in hotspots of HA after permeation of **7**. The CH_2 IR band in the 2850 cm^{-1} range assigned to lipid acyl chains is specific of the organization of lipids,[39, 40] and the amide I band can be analyzed to probe a modification of the proteins structure.[41] Spectra were recorded at the surface of the skin exposed to **7** for 24 hours, at some points with various intensities of the A_1-band and at the surface of the skin not exposed (Figures S11 and S12, SI). No variation in peak position was seen at 2850 and 1500-1700 cm^{-1}, which suggests that **7** does not induce any modification of the organization of lipids or proteins.

In conclusion, we were able to efficiently label HAs of different MW with a $Re(CO)_3$ SCoMPI at different rates using a Ugi reaction. Their penetration into human skin biopsies was monitored using IR and fluorescence microscopies. The label percentage was an important parameter to ensure detection without modifying to a too large extent the physico-chemical properties of the biopolymer: if too low, the compound (**6**, 10 kDa, 3-4%) was not seen, but when too high (**8**, 10 kDa, 40% and **9**, 400-1000 kDa, 20%), solubility was impaired and hence penetration as well. After a 7-hour exposure, **7** (10 kDa, 20%) was localized at some spots in the SC (Figure S9), whereas **10** (400-1000 kDa, 5%) was not detected (not shown). After a 24-hour exposure, **7** was homogeneously distributed in the SC and slightly in the VE (Figure 2), but was not detected in the deeper layers of the skin whereas **10** was only located at some

spots in the SC (Figure S9). The present data showed that the penetration of a cosmetic relevant compound in the skin can be efficiently probed using a SCoMPI. As expected, the penetration is highly dependent on the size of HAs, with no modification of the skin structure.

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Supporting Information
The following file is available free of charge: supporting information about synthesis, methods and additional data (PDF).

Abbreviations
HA, hyaluronic acid; SC, stratum corneum; VE, viable epidermis; MW, molecular weight; (SR)-FTIR-SM, (synchrotron radiation) Fourier transform infrared spectromicroscopy; ROI, region of interest; SCoMPI, Single core multimodal probe for imaging; DIPEA, diisopropylethylamine; TFA, trifluoroacetic acid

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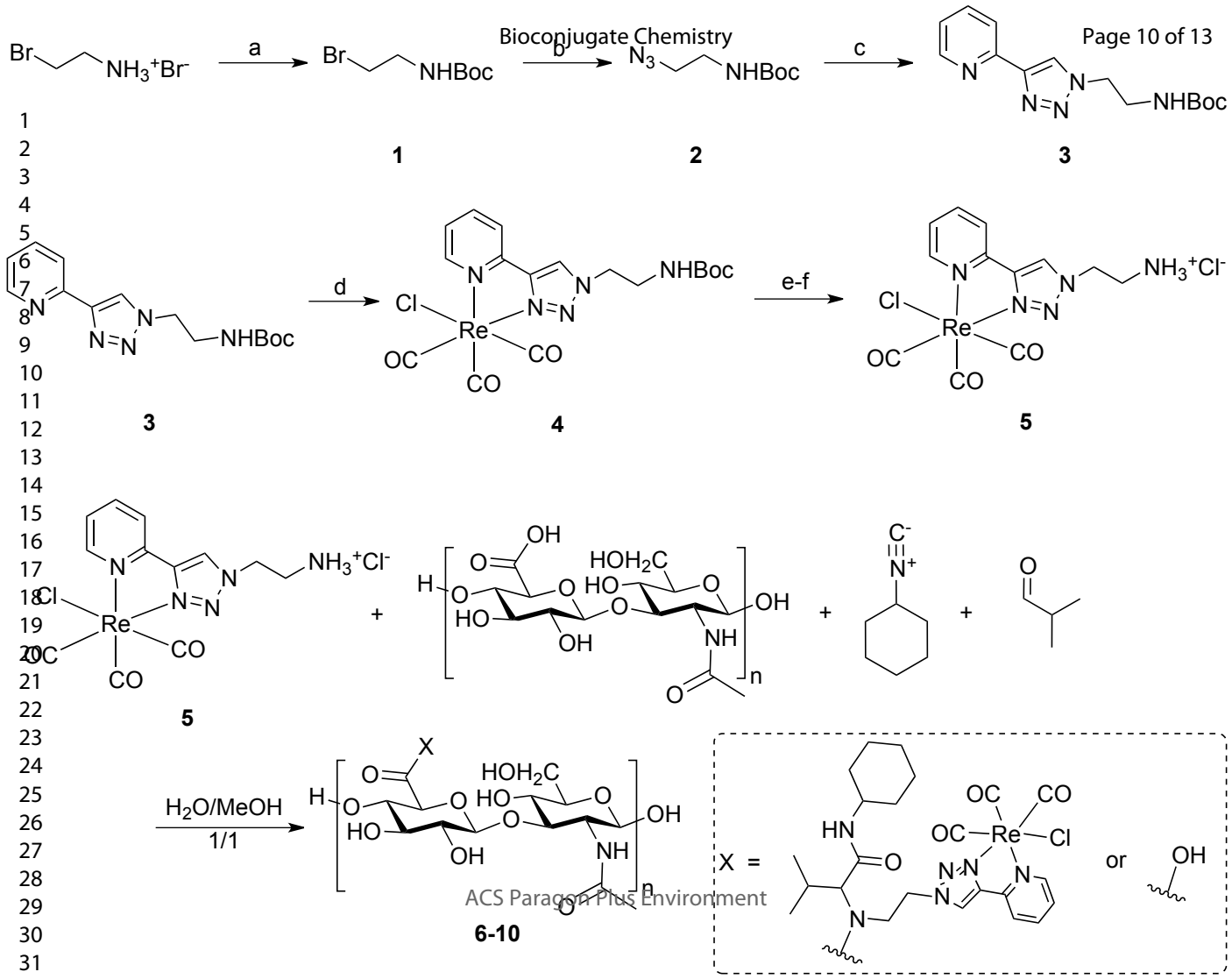

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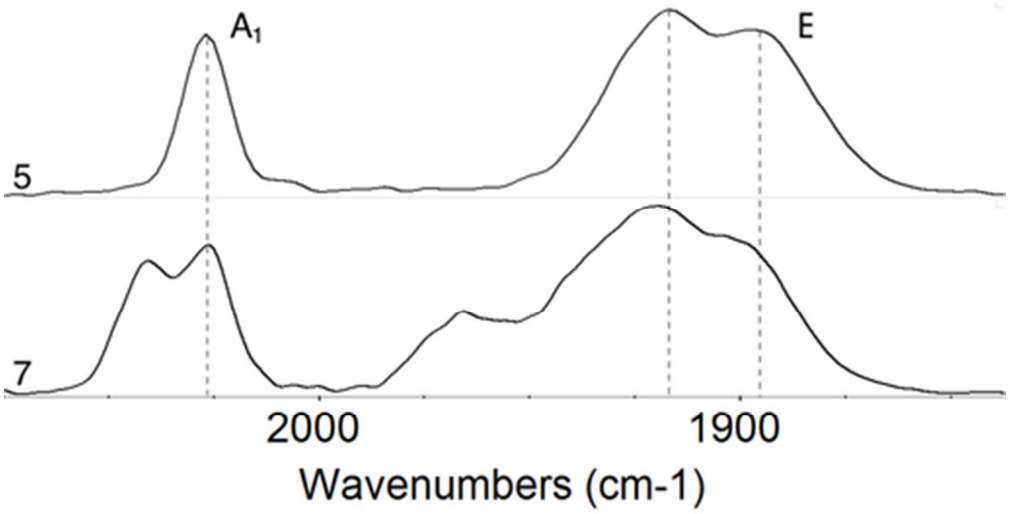


Figure 1. IR spectra obtained using ATR mode between 1820 and 2075 cm-1 of 5 in ethanol and 7 in water (2 mg·mL-1)

43x22mm (300 x 300 DPI)

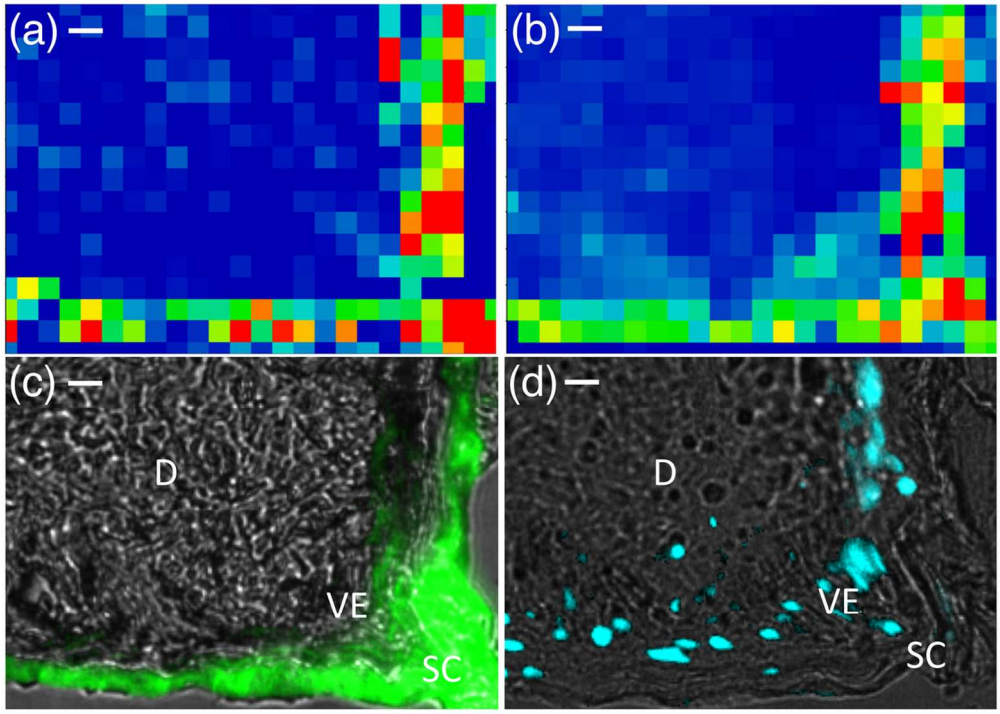
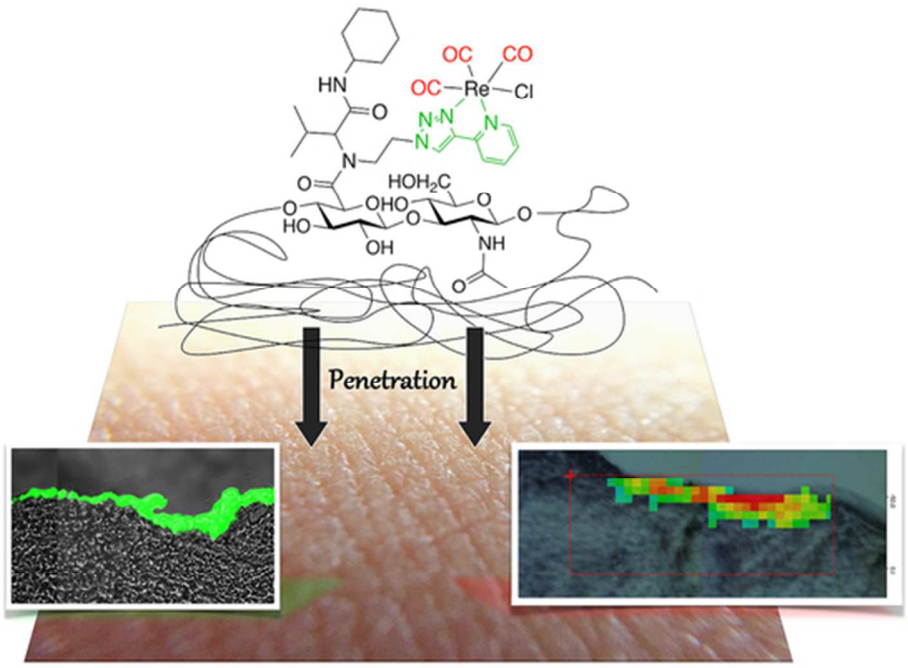


Figure 2. Map of a 10 µm-thick skin cryosection after a 24-hour exposure with 7, mounted on a CaF2 window (scale bar = 10 µm) (a-b) SR-FTIR-SM images based on the integration of absorption bands: (a) A1-band (2055-2005 cm-1), (b) CH2 (2868-2838 cm-1), using a false color scale from blue (low) to red (high intensity), (c, d) Bright field image merged with (c) the luminescence signal of 7 (ex 350/50x, em 560/80m) and (d) nuclei staining (Hoechst, blue, ex 350/50x em 460/50m); 128 scans, 8 cm-1 spectral width. D=dermis, VE=viable epidermis, SC=stratum corneum.

60x43mm (600 x 600 DPI)



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50x33mm (300 x 300 DPI)