Re(I) carbonyl complexes: Multimodal platforms for inorganic chemical biology

Sarah Hostachy, Clotilde Policar, Nicolas Delsuc

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Abstract

Bio-imaging, by enabling the visualization of biomolecules of interest, has proved to be highly informative in the study of biological processes. Although fluorescence microscopy is probably one of the most used techniques, alternative methods of imaging, providing complementary information, are emerging. In this context, metal complexes represent valuable platforms for multimodal imaging, since they may combine interesting spectroscopic features and biologically relevant functionalization on a single molecular core. In particular, d^6 low-spin rhenium tri-carbonyl complexes display unique luminescence and vibrational properties, and can be readily functionalized. Here we review their applications and potential as probes or drugs relying on their photophysical properties, before focusing on their use as multimodal probes for the labelling and imaging of peptides and proteins.

Introduction

Fluorescence microscopy is one of the most widely used techniques for the visualization and study of biological processes. Its good spatial resolution (a few hundreds of nanometers, ie ~ $10^2 \times 10^2$ nm^2 for conventional microscopy experiments) makes it well suited for sub-cellular imaging, since typical eukaryotic cell diameters are in the range of a few tens of micrometers. In addition, due to its good spatial resolution, fluorescence imaging in live cells is achievable, providing relevant dynamic information. A large set of fluorescent probes with high quantum yields and diverse excitation and emission wavelengths are available, thus enabling sensitive and multi-color imaging. Although highly valuable, fluorescence techniques also suffer from some drawbacks, such as photobleaching and photodamage of cells. Moreover, the fluorescence quantum yield of a probe is often sensitive to its environment (pH, hydrophobicity, polarity) and self-quenching can occur, for instance in the case of local accumulation of the probe (e.g. in a membrane). This means that intensity of fluorescence do not always correlate with quantity, which generally makes reliable quantification difficult. Ratiometric systems are proposed in the literature to solve these issues, but the question of the possible dependence on the relative quantum yields with environment and self-quenching extinction remains.

On the other hand, complementary methods for bio-imaging have recently emerged. Vibrational techniques such as infrared (IR) or Raman microspectroscopies have raised a particular interest over the past few years. IR spectroscopy and IR-imaging involve excitation in the vibrational levels. For fluorescence, excitation in the electronic levels is associated with modification in the electronic distribution and hence in the reactivity of the molecule: chemical reactions can thus occur, with possible photobleaching of the probes. In contrast, when vibrational levels are excited in the fundamental electronic state, the only dissipation is through the release of heat and thus no photobleaching occurs. In addition, IR microspectroscopy can be used to get chemical information about biological samples through signatures of lipids, esters, amides, or DNA [1–3]. The deeper penetration of IR radiation, in comparision with higher energy light, also enables tissues imaging. However, infrared microspectroscopy generally displays lower spatial resolution than fluorescence microscopy. In optical spectroscopy, the spatial resolution is limited by diffraction to $\sim \lambda/2$ (Abbe diffraction limit), where λ is the light wavelength. For infrared microscopy, typical spatial resolution is thus in the 1–10 μm range. To overcome this limitation, near-field techniques in the IR regime have been implemented. Combining scanning probe microscopy with spectroscopy is a way to circumvent the limitations inherent to optical detection and to record highly spatially resolved spectral information: the spatial resolution is then mainly governed by the nanometric probe [4,5]. In this field, scattering scanning near-field optical

microscopy or s-SNOM [6] and atomic force microscope infrared (AFMIR) spectroscopy have been employed on biological samples – including cells - and enabled reaching resolution below 100 nm by using an atomic force microscope (AFM) tip [7–10]. By recording IR spectra at given locations of cells or tissues, IR microspectroscopy enables information-rich mapping of biological samples. However, this information comes at a cost, which is often the temporal resolution of this mode of imaging: fixed samples, with long recording time, are generally used in order to obtain a good spatial resolution. Nevertheless, IR imaging of live cells is possible and has been successfully performed [11].

In this context, multimodal probes are thus attractive, as they combine the advantages of the different methods of imaging on a single molecule. Such probes can be used as tags if they can be conjugated to a molecule of interest (MOI). To reach that goal, a well-documented strategy consists of combining several modalities by covalently linking to a scaffold a molecular moiety dedicated to each probing modality. This leads to high molecular weight compounds, which require multi-step synthesis; if use as tags, they may alter the cell penetration of the MOI to large extent. The molecular construct may also be disrupted in the biological environment, which would result in inconsistent images in the various modalities. Alternatively, metal complexes bearing organic ligands may be particularly adapted to obtain multimodality, and the possible decoration of the ligand enable the combination of unique spectroscopic properties and of biologically relevant functionalization on a single molecular core. Re(I) tri-carbonyl complexes, in particular, display interesting luminescence properties (large Stokes shifts, long luminescence life-time) and intense absorption bands (1800–2200 cm^{-1}) in the IR transparency window of biological samples. Indeed, their use for correlative fluorescence and infrared imaging was recently exemplified which demonstrates their validity as Single Core Multimodal Probes for Imaging (SCoMPIs) [10,12–14]. Biological applications of $Re(CO)_3$ complexes based either on their luminescence or infrared properties have been extensively reviewed [15–25]. In the present review, after a brief overview on their applications and potential as probes or drugs relying on their photophysical properties, we will attempt to give a special focus on their use as multimodal probes for the labelling and imaging of peptides and proteins.

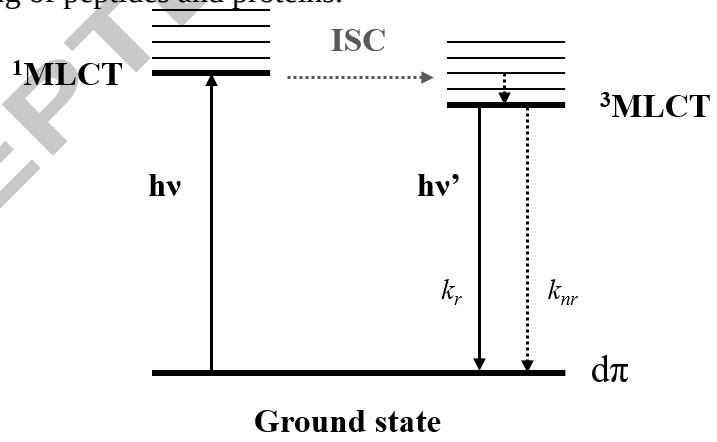


Figure 1. – Simplified Jablonski diagram for [$Re(CO)_3$(diimine)(X)] complexes (MLCT model). Solid arrows represent radiative processes, dotted arrows represent non radiative processes.

1. $Re(CO)_3$ complexes in biological studies

1.1. Spectroscopic properties of luminescent Re(I) tricarbonyl complexes

Re(I) *fac*-tricarbonyl complexes bearing low energy π^* orbitals (e.g. α-diimine or dipicolylamine-derived ligands) display attractive luminescence properties and biocompatibility features (Figure 1). Similarly to other luminescent d^6 complexes (including luminescent Ir(III) and Ru(II) complexes), low-spin, 18-electron Re(I) tricarbonyl complexes are kinetically inert, which limits ligand exchange and the

toxicity associated with the release of heavy metal ions [25]. The luminescence properties of $[Re(CO)_3(N^N)X]$ complexes (L = α-diimine, X = halide, pyridine...), e.g. low quantum yield, large Stokes shift and long emission lifetime, are typical of ^{3}MLCT excited states: upon irradiation, one electron is promoted from a metal-centered dπ orbital to a ligand-centered π^* orbital (^{1}MLCT excited state). Inter-system crossing (ISC) then leads to the emissive ^{3}MLCT excited state.

However, halide-to-ligand charge-transfer (^{3}XLCT) or ligand-to-ligand charge-transfer (^{3}LLCT) may also occur, as well as mixing with intraligand ^{3}IL ($\pi \rightarrow \pi^*$) states [20,26,27]. Although the nature of their emission is thus phosphorescence, these complexes are widely referred to as luminescent in the literature, and we will use this terminology in the following. Finally, the emission properties of Re(I) complexes are often sensitive to the presence of triplet quenchers like $^3O_2$. Although interaction with $^3O_2$ results in emission intensity loss (quenching), this mechanism does not chemically alter the complex, and is thus distinct from photobleaching [25].

Due to their photophysical features, Re(I) tricarbonyl have raised an increasing interest for fluorescence bioimaging applications. Several cellular and biomolecular probes based on these complexes have thus been developed. In particular, efforts were made - and still are - to improve the spectroscopic features of these complexes, i.e. to obtain higher quantum yields and excitation at longer wavelength. This is generally done by tuning the structure of the N^N ligand, since it is involved in the MLCT. Two families of ligand have been particularly explored: (i) polypyridyl ligands, mostly derived from 2,2'-bipyridine (bpy) and 1,10-phenanthroline (phen) [19–21,23–25,28–42] and (ii) bis-quinoline and its derivatives, e.g. bis(phenanthridinylmethyl)amine (bpm) [22,43–45]. However, other polyazaheterocycles have also been developed, such as 1-R-4-(2-pyridyl)-1,2,3-triazole (pyta) and its derivatives [46–48]. More recently, Re(I) tricarbonyl complexes incorporating 5,5'-disubstituted 3,3'-bisisoxazole have also been described [49]. Modifications of the X ligand may also alter the luminescence properties of the complex. For instance, for the $[Re(CO)_3(bpy)(X)]$ complex, substituting the chloro for a pyridine (py) has an impact on both emission wavelength and quantum yield (Figure 2, Table 1).

Spectroscopic properties of some rhenium complexes are summarized in Table 1. Interestingly, varying the bidentate or ancillary ligands may have a strong influence on quantum yield or emission wavelength. Excitation wavelength, on the other hand, is generally in the 340–360 nm range, which is not favorable for excitation of biological tissues. Indeed, most lasers available on confocal microscope offer excitation wavelength over 400 nm and more energetic lasers induce photodamages. Imaging $(L)Re(CO)_n$ remains possible thanks to the large bandwidth of their absorption band. Interestingly, though, Re(I) tricarbonyl complexes bearing a bis-quinoline ligand could be imaged in cells by fluorescence microscopy with excitation at 488 nm [50,51].

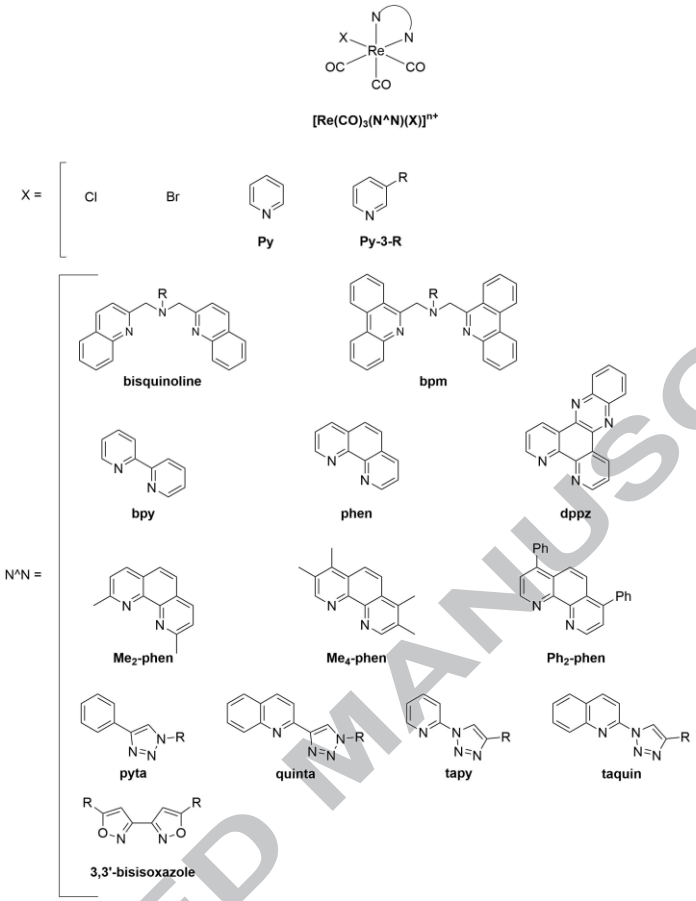


Figure 2. Selection of ligands tested to tune the spectroscopic properties of Re(I) tricarbonyl complexes [19,44,47,49].

Table 1. Photophysical properties of selected Re(I) tricarbonyl complexes in acetonitrile at 298K.

N^N ligand	X ligand	λ_{exc} (nm)	λ_{em} (nm)	Φ_{em} (%)	Ref.
bisquinoline[a]	-	366	425, 580	0.44	[43]
bpm	-	350	570-575	N.A.	[44]
bpy	Cl	355	633	0.27	[46]
bpy	py-3C(O)NH_2	343	551	15.6*	[29]
phen	py-3C(O)NH_2	368	546	12.2*	[29]
phen	py-3C(O)NHEt	355	548	33	[36]
Me_2-phen	py-3C(O)NHEt	355	536	30	[36]
Me_4-phen	py-3C(O)NHEt	355	515	54	[36]
Ph_2-phen	py-3C(O)NHEt	355	560	34	[36]
dppz	py-CH_2-NH-biotin	>350	556, 599 sh	0.16	[42]
pyta (R = Bn)	Cl	355	538	0.33	[46]
pyta (R = alkyl)	Cl	332	522	0.1	[47]
quinta	Br	N. E.[b]	N.E.[b]	-	[47]
tapy	Cl	360	569	0.19	[47]

tapy	Br	360	564	0.17	[47]
taquin	Br	380	617	0.06	[47]

*Measured in degassed acetonitrile. All other measurements were made in aerated solution or it was not specified if the measurement was in degassed acetonitrile.

[a] Measurements were made in aerated ethylene glycol instead of acetonitrile. [b] $[Re(CO)_3(quinta)(Br)]$ complexes are non emissive in acetonitrile but display typical MLCT features in aqueous solution containing DMSO. Typical photophysical data for those complexes are λ_{exc} = 335-345 nm, λ_{em} = 580-600 nm and Φ = 0.2-0.6% [47].

1.2. Re(I) tricarbonyl complexes with specific cell localization

Owing to their luminescence properties, Re(I) tricarbonyl complexes have been imaged in biological contexts and in particular in cells. A great attention has been paid to the development of Re(I) complexes targeting the different cell compartments, that can be used as organelles trackers. A thiol-reactive chloromethyl group was, for instance, appended to a Re(I) tricarbonyl complex (**1**, R=Cl, Figure 3) in order to immobilized the complex into the mitochondria through its reaction with reduced thiols [39]. Indeed, compound **1** (R=Cl) was found to co-localize with TMRE (tetramethylrhodamine ethyl ester), a known marker of mitochondria, in MCF-7 human breast cancer cell line (Figure 4.1). This organelle specificity is thought to be due to the greater transmembrane potential of the inner mitochondrial membrane that attract positively charged lipophilic molecules. A similar complex bearing a hydroxymethyl group (**1**, R = OH) displayed a very similar localization pattern in MCF-7 cells, despite the apparent lack of a thiol-reactive group, supposedly necessary to anchor the complex to the organelle [40]. To explain this, it was postulated that phosphorylation of the internalized complex could lead to a thiol-reactive moiety, although no experimental evidence for this hypothesis was obtained. Coogan and co-workers synthesized a range of Re(I) tricarbonyl polypyridyl complexes varying in charge, size and lipophilicity in a first attempt to rationalize the cell localization of such compounds [40]. Although rationalization proved sometimes difficult, they could observe for instance that compound **2** (Figure 3) was localized at the plasma membrane of MCF-7 cells, probably interacting with the cationic residues of the glycoproteins layers (glycocalyx). Additionally, compound **3** (Figure 3) was found to be internalized only in dead or damaged cells, being thus a potential cell-death marker. Examples of biotinylated $Re(CO)_3$ polypyridyl complexes (compounds **4, 5**, Figure 3) were found to localize in lipophilic compartments like Golgi apparatus or endoplasmic reticulum [37,52].

A few years ago, Policar et al. developed $Re(CO)_3$ complexes for correlative imaging. This led to the concept of Single Core Multimodal Probes for Imaging (SCoMPIs) [12]. The idea underlying the single core concept is that both modalities (IR and luminescence) are carried by the same molecular moiety, and are thus closely linked. Indeed, disruption of Re-CO bond(s) upon irradiation for instance, leads to a disappearance of the luminescence signal is observed (unpublished data). On the other hand, the energy of CO vibration is closely related to the nature of the entire $(L)Re(CO)_3$ core, and if L is exchanged a shift in the ν(CO) bands will likely be observed. A Re(I) tricarbonyl complex bearing a 4-(2-pyridyl)-1,2,3-triazole (pyta) ligand appended with a long alkyl chain (compound **6**, Figure 3) was synthesized, and incubated with MDA-MB-231 cells, another breast cancer cell line [12]. The azide moiety was used for comparison of the infrared signals from N_3 and CO groups inside cells. The compound could be detected by both fluorescence and infrared microspectroscopies, with consistent perinuclear localization in both imaging modes, Figure 4.2. The precise location assignment of the SCoMPI was performed through colocalization experiments by fluorescence, which revealed that **6** was accumulated into the Golgi apparatus.

Very recently, Metzler-Nolte *et al.* designed rhenium tricarbonyl complexes bearing asymmetric tridentate ligands derived from the bis-phenanthridine scaffold (compounds **7**, symmetric, and **8-9**, assymetric) [53]. The resulting asymmetric complexes had enhanced luminescence properties, in particular longer emission wavelength (>600 nm in water) and good quantum yields in water for such complexes (around 0.15-0.2%). In cells, these complexes displayed a perinuclear localisation. Colocalisation studies with known markers of the different cell organelles showed that the complexes were found in the endoplasmic reticulum, but not in the Golgi nor in the mitochondria. This was confirmed in five different cell lines. Of note, they observed that the organic organelle stains could show dramatic photobleaching upon prolonged irradiation, whereas the complexes showed stable luminescence intensity throughout these photobleaching experiments.

Finally, a few examples of localization in the nucleus (compounds **10,11**, Figure 3) can also be found [54,55]. It should be noted that for compound **10**, the luminescence comes from acridine rather than from the Re core.

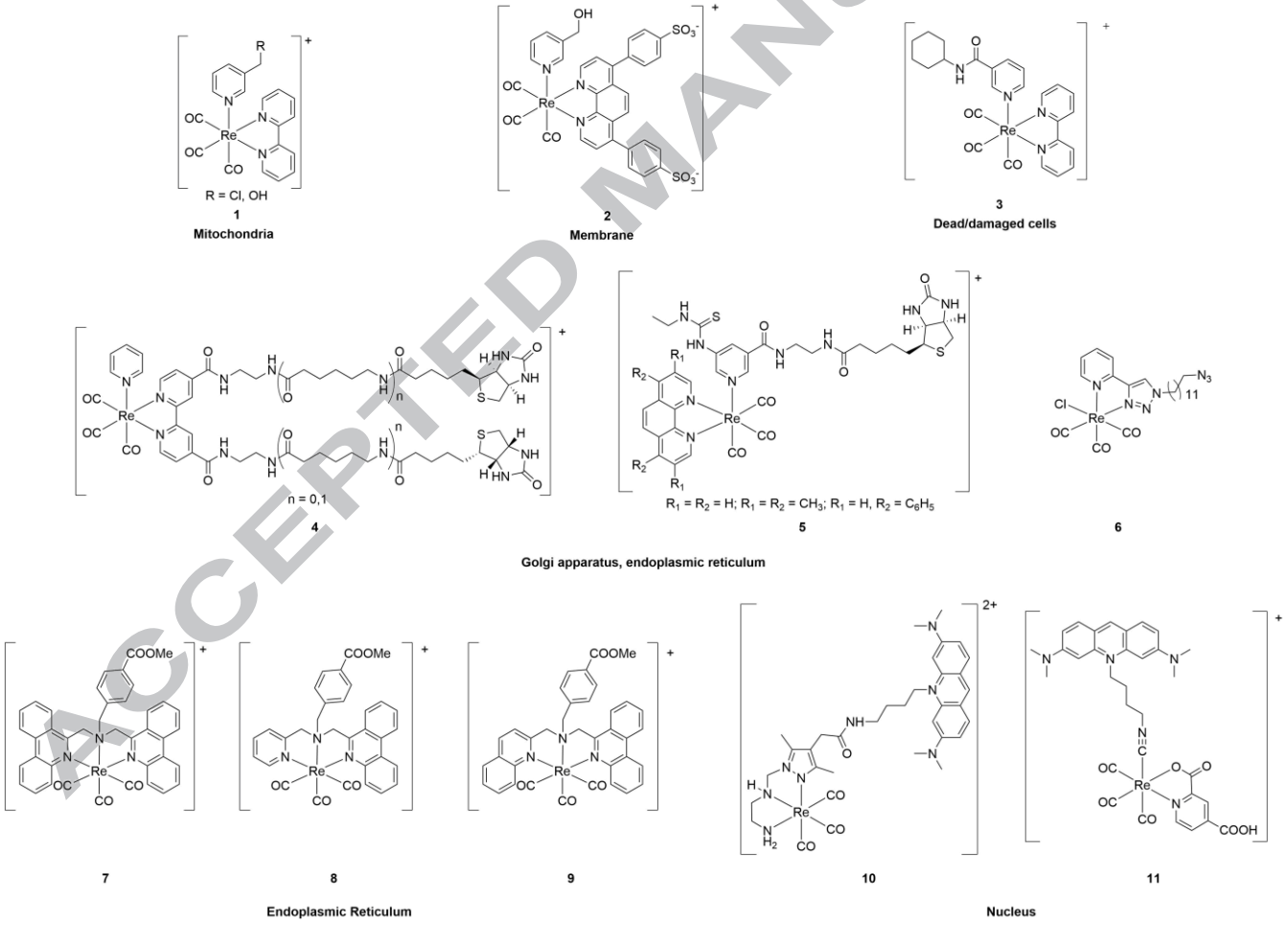


Figure 3 Examples of luminescent Re(I) tricarbonyl complexes targeting the different cell compartments [12,19,37,39,40,45,54,55].

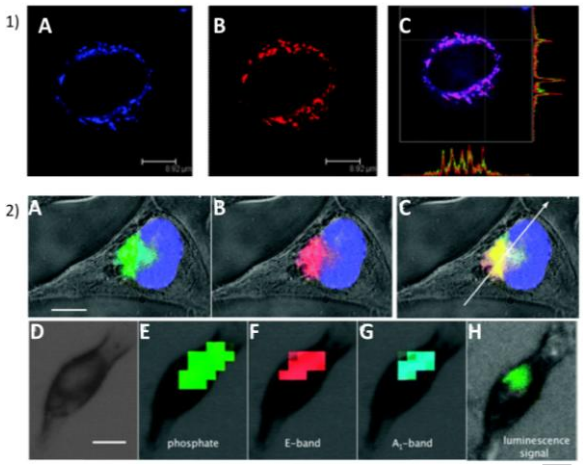


Figure 4. 1) Colocalisation of **1** and TMRE in MCF-7. A **1**. B TMRE C. Merge and cross section adapted from ref [39]. 2) MDA-MB-231 cell incubated with **6**. Bright field image (scale bar 10 μm). A merge of **6** (green) and DAPI (blue). B merge of the Golgi tracker (red) and DAPI (blue). C merge of **6** (green) and the Golgi tracker (red) with their overlay in yellow and DAPI (blue). D Bright field image. E-G SR-FTIR mappings (pixel size: 3 × 3 μm²): E phosphate asymmetric stretching (green), F E-band (red), G A_1-band (cyan). H Epifluorescence image, adapted from ref [12].

1.3. Photoresponsive Re(I) complexes

Beside their use as inert luminescent probes, Re(I) tricarbonyl complexes are attracting an increasing interest as potential photoresponsive drugs. Here we summarize some of their applications as CO-releasing molecules or as photosensitizers for photodynamic therapy.

1.3.1. CO releasing Re(CO)x complexes

CO is endogenously produced through heme degradation by the heme oxygenase (HO) enzyme [56]. At low doses, CO was recently shown to play beneficial roles in various physiopathological situations such as inflammation and oxidative stress or during ischemia-reperfusion [57–59]. Controlled CO delivery is therefore of importance and attracting an increasing attention, and can be rationalized by the electronic structure of CO and M-CO, as detailed below.

CO bears an electronic extra density on the C and not on the O, which explains why CO binds to metal ion by the carbon atom and not the oxygen atom. The Lewis structure of CO (see Figure 5) is unusual because the electronic density lies on the less electronegative atom. This is due to the fact that the HOMO in the CO molecular orbitals diagram is a quasi non-bonding orbital resulting from a 3-orbital interaction which shows a higher atomic orbital coefficient for the $2p_C$ atomic orbital than for the $2p_O$. An important feature for CO reactivity is the presence of a low-lying π* level (2π in Figure 5), antibonding between the C and O atoms. Both the frontier orbitals (HOMO 3σ and LUMO 2π) can interact with a d-orbital from a metal center to create a M—CO bond. The primary interaction is with the 3σ, which shows a very strong density on the C atom, and to maximise the overlap with the d-orbital (dz^2 is exemplified on Figure 5), a linear geometry M—C—O is favoured. The LUMO 2π can also efficiently interact with a metal d-orbital of appropriate symmetry (exemplified in the case of d*xy* in Figure 5). This interaction, called back-bonding, contributes to increasing the strength of the M-CO bonding with a weakening of the C-O bonding. The MCO Lewis structure can then be written with two mesomeric forms, one involving a single M-C bond and the other a double M=C bond as shown in Figure 5, the M-C bonding strength increasing at the expense of the C-O strength. The more electronic density in the d-orbital, the more efficient the back-bonding process and the stronger the M-C bond. Hence, any decrease in the d-electronic density will be associated with a weakening of the M—CO bond and can trigger CO release. Oxidation, or excitation in the MLCT band —which promotes one

electron from the d-manifold to the excited level mainly localized on the ligand, or modification of a ligand becoming less electro-donating to the metal, can trigger release. The rationale for the design of CORMs based on this principle has been recently described in an excellent review [60].

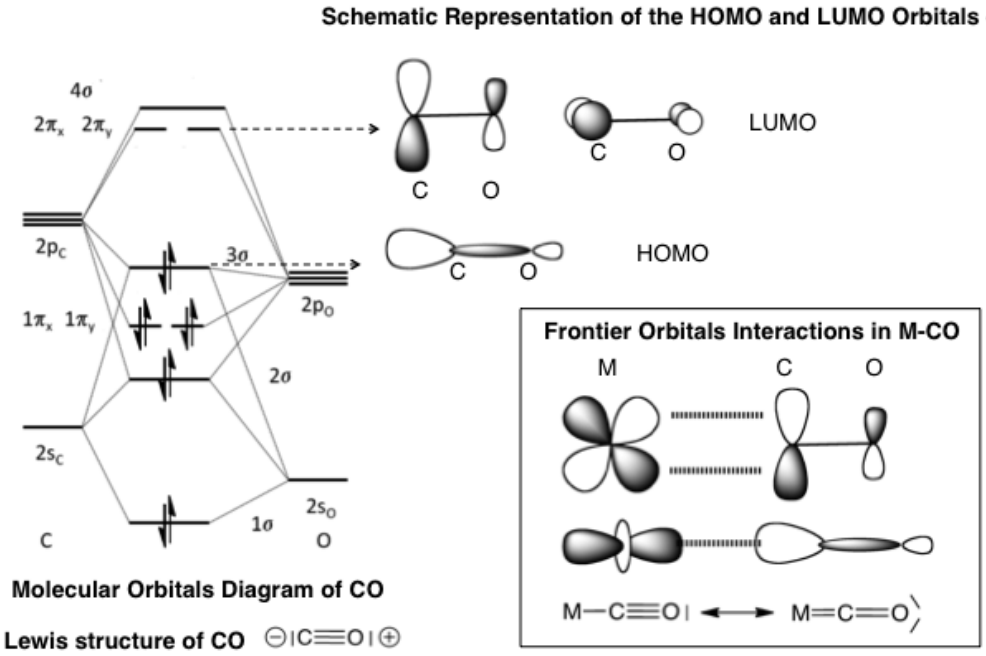


Figure 5. CO Molecular Orbitals and M-CO bonding. Inspired from [60].

In a pioneering work, Motterlini et al. used metal-CO complexes called CO-RMs (CO-releasing molecules) considered as a condensed reservoir of CO or a "solid form of CO gaz" (personal communication with Dr. Motterlini): $Mn_2(CO)_{10}$, $[Ru(CO)_3Cl_2]_2$ and $Fe(CO)_5$ can be used as prodrugs to release CO, spontaneously or upon a specific trigger [61]. Now, many examples based on V, Cr, Mo, W, Co, B and Re bearing organic ancillary ligands are reported in the literature [62]. Among them, uncommon monomeric 17-electron dicarbonyl Re complexes were developed (compound **12,** Figure 6) by Zobi et al. The CO-releasing rate could be modulated by tuning the pH (half-lives shorter at lower pH) and by varying the ligand [63]. However, these complexes were poorly soluble in aqueous media and a second generation conjugated with cyanocobalamin (a vitamin B_{12} derivative, compound **13,** Figure 6) has been studied and showed a protective effect in an ischemia-reperfusion injury model [64]. Efforts have also been devoted to the development of photo-activable CO-RMS. For example, Ford and co-workers have developed a rhenium-based non cytotoxic water-soluble photoCO-RM $[Re(bpy)(CO)_3(thp)]$, (compound **14,** Figure 6) which combines luminescence properties and CO photo-releasing ability [65]. The release of one CO induces a red-shift of fluorescence emission maximum of compound **14**. After incubation in human prostatic carcinoma cells (PPC-1), the internalization of the complex into the cytoplasm was observed by fluorescence microscopy. Upon irradiation at 405 nm, the release of CO and its replacement by a water molecule was evidenced since the emission intensity decreased in the 465-495 nm window and strongly increased at higher wavelengths (> 660 nm) (Figure 7). Although very interesting, the main limitation of this complex relies on the fact that high-energy light is required to promote CO release. In an attempt to increase the excitation wavelength of Re-based photo-CORMS, Mascharak et al. have developed Re complexes exhibiting strong MLCT bands in the visible region, compound **15,** Figure 6 [66]. However, CO release was not observed upon illumination in the visible

region, the absorbed energy being dissipated through pathways that do not initiate CO release. This highlights the difficulty in tuning the properties of Re-based photoCORMS.

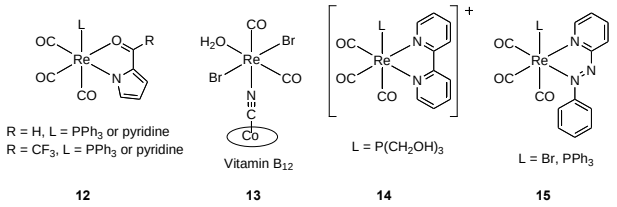


Figure 6. Selected examples of CO releasing Re(I) carbonyl complexes [63–66].

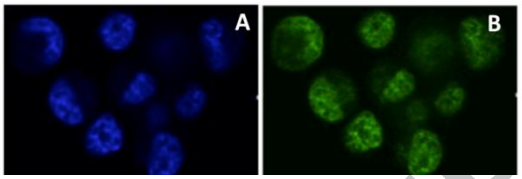


Figure 7. Confocal fluorescence microscopy images of PPC-1 cells that were incubated for 60 min with 50 μM of **14**. The left image (in blue, λ_{em} = 465–495 nm) was collected with minimal photolysis from the 405 nm excitation source and indicates the incorporation of **14** into the cellular cytosol. The right image (in green, λ_{em} > 660 nm) was collected after 405 nm photolysis for 15 min and indicates the transformation of **14**. Adapted from ref [65].

1.3.2. Rhenium based complexes for Photodynamic therapy (PDT)

Photodynamic therapy relies on the use of a non toxic photosensitizer which, upon irradiation and in presence of dioxygen, will generate toxic species. Very frequently, these toxic species are reactive oxygen species (ROS) such as $^1O_2$. This results in oxidative damage of cellular components and then induces cell death. This strategy can consequently find applications in the treatment of cancers or infections. Generally, the sensitizers involved are organic dyes but some transition metal complexes have been used [67]. Although Ru(II) polypyridyl species have been shown to be efficient photosensitizers for $^1O_2$, there are only scarce examples of use of Re(I) complexes for this application. In a pioneering work, Abdel-Shafi and co-workers studied three Re carbonyl complexes bearing either a 1,10-phenanthroline, a 2,2'-bipyridine, or a benzo[h]-quinoline ligand [68] and showed that these complexes were good sensitizers for singlet oxygen. More recently, others complexes with improved properties (higher irradiation wavelengths, higher absorption coefficient…) have been developed [69]. Some of these complexes could even be embedded in a flexible polymeric silesquioxane (SSO) film while retaining their photophysical features, which opens up the possibility to use such films as biological or microbial-photoinactivating surfaces [70]. The Meggers' group designed complex **16** (Figure 8) and tuned the N^N and the R_1 ligands in order to be able to irradiate the complexes with either visible light [71] or red-light [72]. In both cases, the complexes were incubated on HeLa cells and were found cytotoxic after irradiation only, with IC_{50} in the nanomolar range. In addition, cytotoxicity of one of the complexes was also shown in 3D models: melanoma spheroids incubated with the complex and irradiated displayed cell death and loss of spheroid integrity at their edges [71]. As discussed later in this review (Section 2.2), Gasser *et al* also designed $Re(CO)_3$ complexes and peptide conjugates for PDT [73]. These examples illustrate the potential of Re carbonyl complexes in PDT, so far understudied.

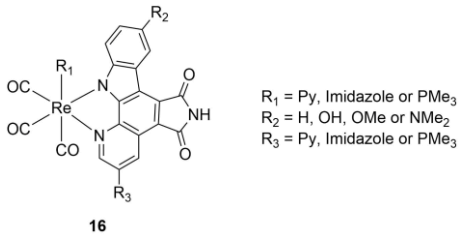


Figure 8. Family of Re(I) tricarbonyl complexes used for PDT and that necessitates low energy irradiation (UV-vis or red light) [71,72].

1.4 Re(CO)x complexes for vibrational imaging

Beside their interesting properties in the UV-visible region, as mentioned earlier, metal-carbonyl complexes present attractive infrared properties, with intense absorption bands in the 1800–2200 cm^{-1} range. There is little IR-absorption from the biological sample in this energy range, which is called the transparency region in IR. The first use of these unique properties in a biological context was a Carbonyl Metallo-ImmunoAssay (CMIA) developed by Jaouen's group. CMIAs are non-radioisotopic (as the tracer is not a radiolabel), competitive (as a known quantity of labelled analyte and a known smaller quantity of the corresponding antibody are introduced in the sample to be analyzed) and heterogenous (as a separation step of bound and unbound fractions is required prior to quantification) immuno-assays, where the tracer conjugated to the analyte for quantifications is a metal-carbonyl moiety. They were able to design mono-immunoassays for various molecules of clinical relevance, such as anti-epileptics and hormones, or of environmental interest (pesticides) [15]. Interestingly, when the infrared bands of different $M(CO)_x$ do not overlap, simultaneous immunoassay (multi-CMIA) can be envisioned. In that sense, M(CO) can be used as multicolor probes or markers. Multi-CMIA of compounds **17, 18** and **19** was reported (Figure 9) [74]. Although example of Re carbonyl complexes has not been yet reported for such assay, their IR properties perfectly meet all requirements and they can be considered as potential candidates for CMIAs.

More recently, Policar et al. have tagged a mestranol derivative **20** (Figure 9) and showed that the labelled molecule could be imaged in MDA-MB-231 and MCF-7 breast cancer cell lines using synchrotron based FTIR microspectroscopy (SR-FTIR-MS), AFMIR and confocal Raman microscopy [10]. This illustrates the potential of Re(I) tricarbonyl complexes as tags for vibrational imaging techniques, including IR and Raman. Interestingly, correlative imaging could also be performed using synchrotron-based UV microspectroscopy (SR-UV-MS), wide field and confocal fluorescence microscopies.

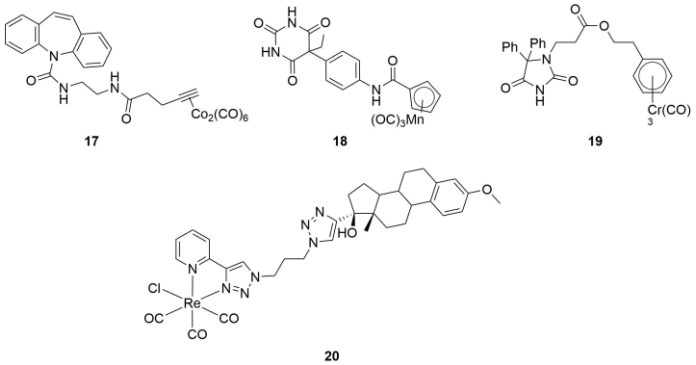


Figure 9. Examples of molecules labelled with metal-carbonyl complexes for Carbonyl MetalloImmunoAssay and a mestranol derivative labelled with a multimodal tag [10,15,74].

Due to the low resolution of optical IR microscopy (see above), IR imaging of cells can be more challenging than fluorescence microscopy. However, the deeper penetration of IR radiation makes IR imaging more suitable for tissues imaging. Recently López et al. used a $Re(CO)_3$ SCoMPI conjugated to a long alkyl chain (C_{12}) to follow the penetration of lipid assemblies into skin by SR-FTIR-MS [75]. In this work, the bicosomes could be easily imaged and also quantified in skin sections using the IR signature of a $Re(CO)_3$ which would not have been straightforward without this tag.

Very interestingly, vibrational imaging is not anymore limited to two dimensions using a standard infrared microscope since Quaroni et al. have implemented a method and protocols to access a quantitative 3D distribution of an exogenous Re carbonyl complex ($Re(CO)_3(H_2O)_3^+$) [76]. This opens up the opportunity to perform computed 3D tomography of cells.

1.5. $Re(CO)_3$ as surrogates for $^{99m}Tc(CO)_3$

Radiotracers and radiopharmaceuticals are widely used in nuclear medicine for diagnosis and treatment. Among radionuclides, ^{99m}Tc displays attractive features, including well-suited properties ($t_{1/2}$ = 6 h, 140 keV) and availability from commercial generators [22,77–80]. Moreover, the use of $[^{99m}Tc(H_2O)_3(CO)_3]^+$ reagent, developed by Alberto and Schibli *et al.*, enables efficient preparation of the radiotracer from any ligand [81]. Finally, non-radioactive isostructural Re(I) tricarbonyl complexes can be prepared and characterized without handling radioactive materials. As a consequence, a large number of protein-specific $Re(CO)_3$ complexes have been developed as *cold* analogs of the *hot* Tc complexes, with the intent to replace Re by its ^{99m}Tc counterpart for the final application. Various proteins have been targeted, including Translocator Protein TSPO [82], metallothioneins [83], G Protein-Coupled Estrogen Receptor GPER/GPR30 [84] or Carbonic Anhydrase IX [85]. As an example, the synthesis of a series of $M(CO)_3$-based (M = Re, Tc) Carbonic Anhydrase IX inhibitors was developed using $Re(CO)_3$, while the affinity for CA-IX was evaluated using the final ^{99m}Tc complexes [77]. Peptides were also labelled with Re/Tc tricarbonyl complexes, generally in order to target specific cell lines or events [86–89]. In most of these examples, the rhenium is used for its reactivity close to the technetium and for the similarities of the Re and Tc complexes. Moreover, as mentioned earlier, efforts are made to use the potential of $Re(CO)_3$ complexes as luminescent probes, and to develop isostructural nuclear and optical probes based on ^{99m}Tc and Re, respectively [22,43,88,90,91]. In addition, ^{186}Re and ^{188}Re isotopes can be used for radiotherapy, and $^{186/188}$Re tricarbonyl complexes have been designed for that purpose [92–94]. Generally, these complexes, luminescent or not, may be inspirational for further development of $Re(CO)_3$ complexes as multimodal imaging probes.

In this first part, a brief overview of the use of Re(I) carbonyl complexes based on their photophysical properties in biological contexts has been given. The next section will focus on strategies developed to specifically label and image peptides or proteins of interest.

2. Labelling of peptides with $Re(CO)_3$ complexes

2.1. Conjugation to single amino-acids and Single Amino-Acid Chelate (SAAC) strategy

Single amino-acids have been derivatized with luminescent $Re(CO)_3$ complexes in order to incorporate them into peptides, or to study their photophysical properties as such in cuvette or in a cellular context [22,43,90,95–97]. Complexes **21** and **22** (Figure 10), for instance, have been appended with phenylalanine and tyrosine, and their photophysical properties were studied [95]. The study particularly focused on the use of the rhenium complex as photo-oxidant to generate tyrosyl radicals by exciting the

MLCT. This fast and controlled generation of tyrosyl radicals may be used to study redox biological processes and in particular electron transfer.

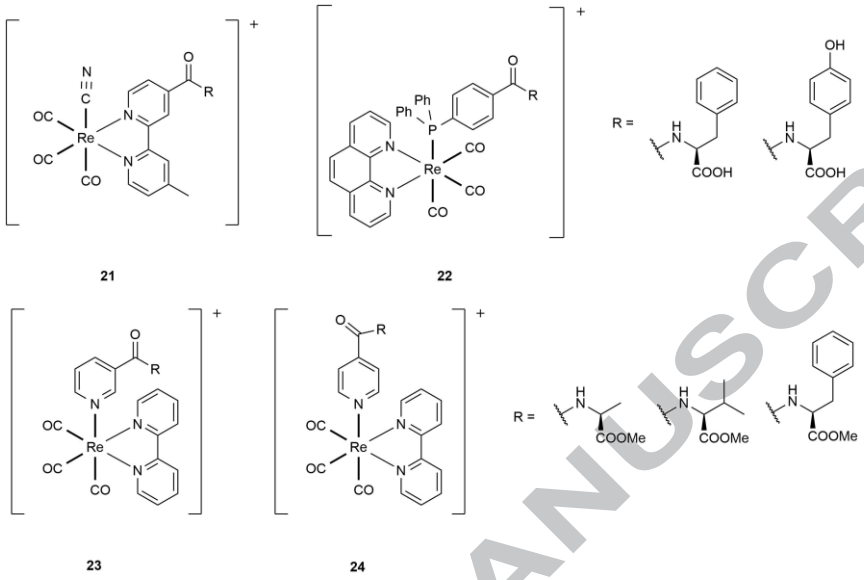


Figure 10. Examples of Re-appended amino acids [95,96].

Gimeno et al. synthesized two series of amino-acids appended with $[Re(CO)_3(bpy)(py)]^+$ complexes (compounds **23** and **24**, Figure 10) [96]. The amino acids were linked to the complex through the pyridine ligand, at either the *meta* **(23)** or *para* **(24)** position on the pyridine. They studied the cellular uptake of both series and could observe that this minor change (*meta* or *para*) in structure could have major consequences on the properties of the complex: the *para* derivatives proved highly cytotoxic and displayed high photobleaching, whereas the cells incubated with the *meta* derivatives remained healthy and displayed localization patterns typical for monocationic, lipophilic Re(I) tricarbonyl complexes (*i.e.* cytoplasmic staining, with some concentration in mitochondria) (Figure 11). Although no explanation could be given as certain, the authors hypothesized that unidirectional electron shuttling from the metal centre to the amino-acid was easier with the *para* than with the *meta* derivatives, generating an amino-acid radical supposed to be involved in an intramolecular deleterious photoinduced reaction with an unidentified biological species.

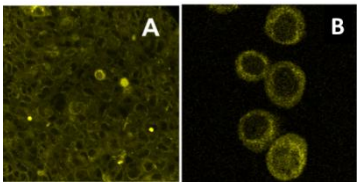
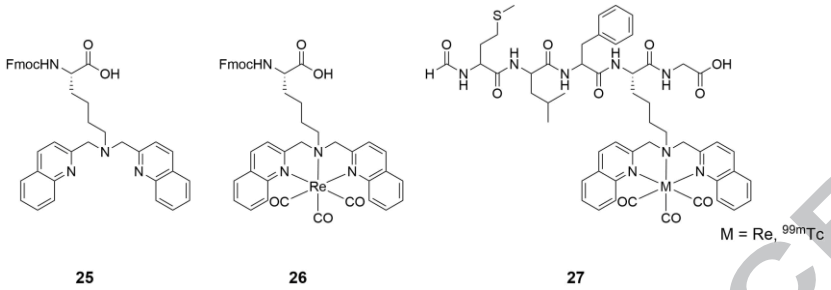


Figure 11. A. Cell imaging with complexes **23** showing clustering. B. Cell imaging with complexes **24** showing localization. Adapted from ref [96].

The single amino-acid chelate (SAAC) strategy was developed in the early 2000s to incorporate Re/^{99m}Tc complexes into peptides by solid-phase peptide synthesis (using Fmoc strategy). Valliant, Zubieta et al. modified a lysine with a bis-quinoline ligand to form the SAAC ligand **25** (Figure 12), which could then be reacted with a $Re(CO)_3$ precursor to form the SAAC-Re complex **26** (Figure 12) [43,98]. Both SAAC and SAAC-Re could be incorporated by automated peptide synthesis into the formylated MLF peptide fMLF peptide (fMLF(SAAC-Re)G, **27**, Figure 12), a peptide sequence

targeting the formyl peptide receptor (FPR). FPR is expressed on neutrophils and is a target for the (radio)imaging of trafficking of white blood cells.



25 26 27

Figure 12. Example of a SAAC ligand and its corresponding complex. This modified amino acid has been incorporated into the fMLF peptide [43,98].

The $Re(CO)_3$-labelled peptide could be observed by fluorescence microscopy in human leukocytes, and its localization was consistent with previous reports on chemotactic peptides (*i.e.* the peptide localized first at the membrane at 4°C and then into the cytoplasm when the temperature was increased to room temperature). The $^{99m}Tc(CO)_3$ analogue could be easily prepared by reacting the precursor $[^{99m}Tc(CO)_3(H_2O)_3]^+$ with the Re-free peptide fMLF(SAAC)G, thus enabling correlative studies in fluorescence and radioimaging. This strategy was later applied to peptides such as HIV-Tat basic domain [88] or β-breaker peptides [99]. Other biomolecules could also be labelled and imaged, e.g. biotin [100,101], folic acid [97] or thymidine derivatives [22,51,102].

2.2. N-terminal labelling of peptide

A Re(I) tricarbonyl complex (**28**), suitable for *N*-terminal labelling of peptides, could be coupled to neurotensin (8-13) fragment on solid-phase (Figure 13) [44]. The complex **28** and the labelled peptide **29** (Figure 13) were both internalized in various cell lines, and displayed cytosolic localization. Uptake of the unconjugated complex seemed more efficient than uptake of the labelled peptide. This observation is general: peptides are not very efficient at enhancing the cell-penetration of molecules that are very hydrophobic or amphiphilic. However they can provide specificity, and peptides may also be conjugated to Re(I) tricarbonyl complexes to target the complex to specific subcellular compartments or to enhance their uptake by specific cells, generally for therapeutic purposes. This was the case for the labeled peptide **29** that exhibited more pronounced differences in its cellular uptake between the cell lines than the parent complex, suggesting some selectivity in its uptake mechanism.

Gasser et al. conjugated Re(I) tricarbonyl bisquinoline compound **30** with a Nuclear Localization Signal (NLS) sequence (**31**) and to a derivative of the Bombesin neuropeptide (**32**) (Figure 13) [73]. Bombesin is often used to selectively target cancer cells over healthy cells, since its receptor is overexpressed in some types of cancers. The aim of the study was consequently to evaluate the potential of Re(I) tricarbonyl complexes as photodynamic therapy (PDT) photosensitizers. Fluorescence microscopy showed that the NLS-conjugated complex **31** accumulated in nucleoli of HeLa cells, whereas its parent complex was homogeneously distributed in the cells. The Bombesin conjugate (**32**), however, displayed only weak fluorescence in those cells, which was explained by either low uptake of the conjugate or quenching of the luminescence of the complex in the cell. To be used in photodynamic therapy, photosensitizers should not be cytotoxic in absence of irradiation. Neither the parent complex **30** nor the Bombesin conjugate **32** displayed cytotoxicity in the dark, whereas conjugation to NLS sequence increased the cytotoxicity of the compound **31**. However, irradiation of the cells incubated with **30** or **32** at 50-100 µM resulted in increased cytotoxicity (up to 20-fold for Re-Bombesin **32**), which is

encouraging for further studies on Re(I) tricarbonyl complexes as photosensitizers. $^1O_2$ production involves non-radiative quenching of the ^{3}MLCT excited state, and thus decreases the effective quantum yield of the complex. Nevertheless, all derivatives could be detected by fluorescence microscopy in live and fixed cells, with the exception of the Bombesin derivative, which can be due to a poor cellular uptake or the quenching of the fluorescence inside cells.

The same group reported the conjugation of a Re(I) tricarbonyl bipyridine complex to a lipopeptide (namely, myristoylated HIV Tat peptide) known for its cell-penetration properties [27]. The conjugate compound **33** (Figure 13) displayed enhanced uptake in HeLa cells (Figure 14), as well as increased cytotoxicity. In another example, Alberto et al. reported the synthesis of an other Bombesin-conjugated Re(I) tricarbonyl complex, compound **34** (Figure 13) [54]. The goal of the study was to design Re/Tc tricarbonyl complexes that could be addressed selectively to the nucleus of cancer cells. The acridine moiety was appended for nucleus targeting, and the Bombesin derivative for cancer cell selectivity. Interestingly, compound **34** could be observed by fluorescence microscopy in PC-3 (human prostate adenocarcinoma) cells, which express the GRP receptor (gastrin releasing peptide receptor) targeted by Bombesin, but was not detected in B16-F1 cells (mouse melanoma cell line), which do not express this receptor. As mentioned above, the complex **11** missing the Bombesin sequence accumulated in the nucleus. However, the Bombesin conjugate displayed homogenous distribution and no nuclear uptake. It was thus hypothesized that the bombesin sequence prevented the nuclear uptake of **34**.

Policar's group labelled cell penetrating peptides (CPP) with SCoMPI and imaged them correlatively by fluorescence microscopy and IR microspectroscopy in skin biopsies (compound **35**, Figures 13 and 14) and in cells (compounds **36**, Figure 13) [14,103]. In cells, they took advantage of the IR modality of the probe to quantify the internalization of the conjugates. They could also show that apparent discrepancies with fluorescence quantification were due to a variable fluorescence enhancement of the probes in membrane environment, which hampered rigorous quantification [13,103–106]. In skin biopsies, the IR modality also enabled them to quantify the penetration of the peptide conjugate into skin, while the fluorescence modality could be used to compare its localization with those of nuclei, using common fluorescence stain like DAPI. More generally, quantification using fluorescence can be difficult due to the dependence of quantum yield with the environment. IR-detection based of the vibrational properties of M(CO) does not suffer from this strong drawback and SCoMPI with both fluorescence and IR are thus quite valuable for reliable quantification [13,103]. The multimodality of the Re(I) complex proved here useful to access information on both quantification and localization.

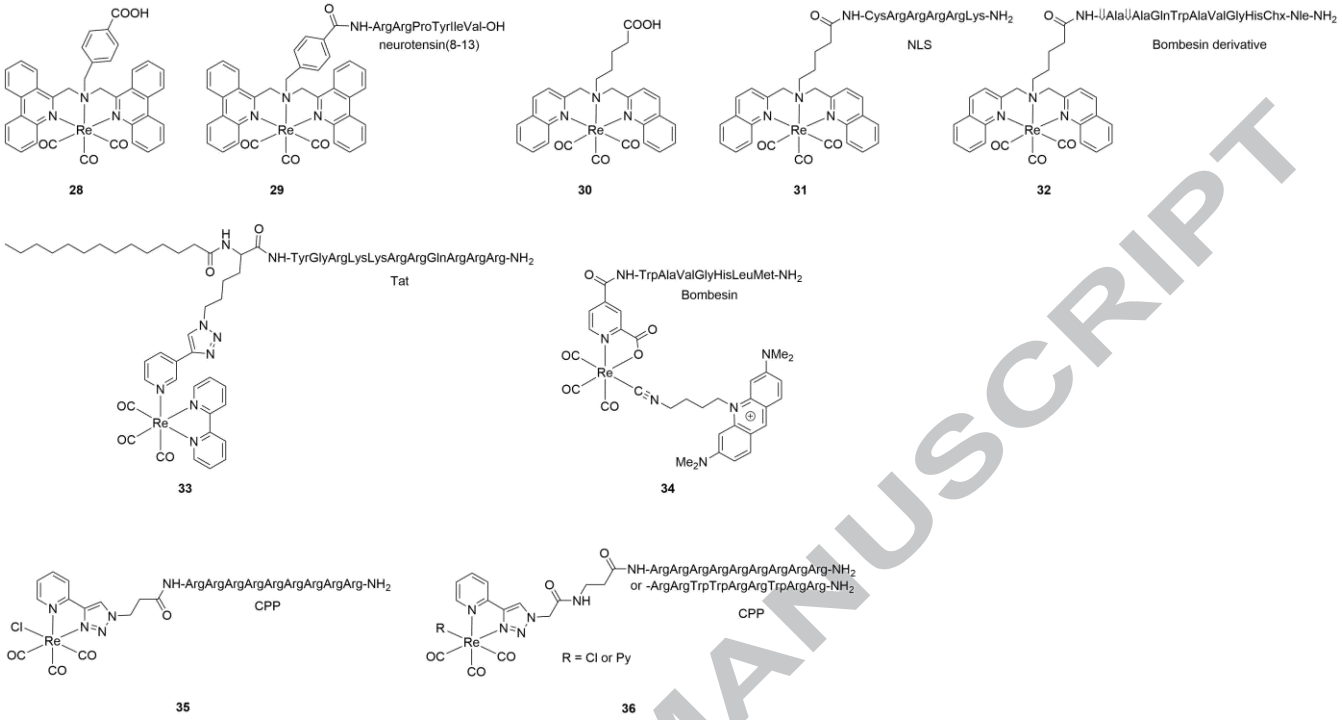


Figure 13. Examples of Re(I) tricarbonyl complexes conjugated to peptide via N-terminal labelling for cell or organelle targeting [14,27,44,51,73,103].

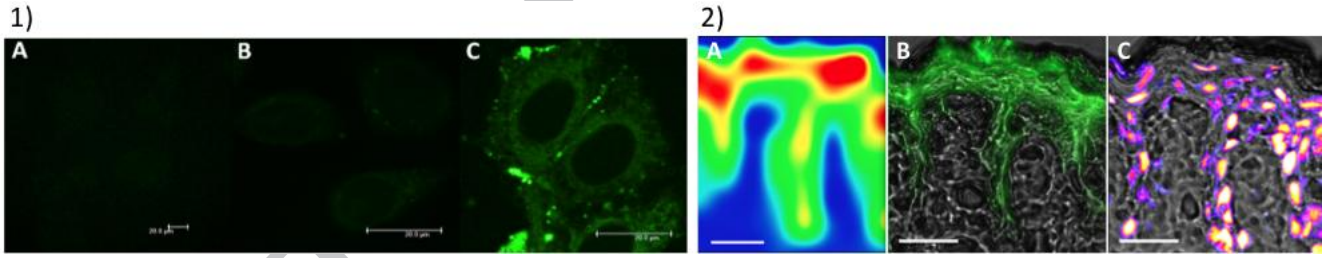


Figure 14. Imaging of N-terminal labelled peptides in cells 1 and in skin biopsies 2. 1) Fluorescence microscopy of HeLa cells fixed after 2 h: (A) untreated cells; (B) treated with $[Re(CO)_3(bipy)(py\text{-}alkyne)](BF_4)$ at 100 µM; and (C) treated with **33** at 20 µM adapted from ref [27]. 2) Skin slice after a 24 h-exposure to a 2 10^{-2} M solution of **35** in water. A. mapping of the integral of the absorbance of the A_1-band (2040-2000 cm^{-1}). B. Bright field image merged with the luminescence signal of 32. C. Bright field image merged with the staining of nuclei by DAPI. Scale bar 20 µm. Adapted from ref [14]

3. Labelling of proteins with $Re(CO)_3$ complexes

As emphasized in this review, $Re(CO)_3$ complexes are interesting IR and luminescent probes for bioimaging. This has motivated the development of $Re(CO)_3$ complexes suitable for the labelling of more complex biomolecules such as proteins. Although the properties of the resulting Re-protein conjugates were mainly studied in cuvette in a first time, these bio-reactive Re complexes are the first step towards the multimodal imaging of proteins in cells.

3.1. In vitro covalent labelling of proteins

Luminescent Re(I) tricarbonyl complexes have been functionalized with thiol- or amine- reactive groups in order to label peptides or proteins *in vitro*. For instance, N-hydroxysuccinimide (NHS)-activated ester **37** (Figure 15) was used to label human serum albumin (HSA) and Immunoglobulin G (IgG) [107]. The authors later used Re-labelled HSA in a Fluorescence Polarization Immunoassay

[108]. The isothiocyanate-functionalized complexes **38** (Figure 15), bearing various bidentate ligands, were coupled to HSA [33]. Similarly, thiol-reactive groups such as iodoacetamide (**39**, Figure 15) and maleimide (**40**) have been appended to Re(I) tricarbonyl complexes. Compound **39** was successfully used to label HSA, with a final dye:protein ratio of 0.7 [109]. Similarly, compound **40**, bearing a thiol-reactive maleimide moiety, was used to label HSA and BSA (Bovine Serum Albumin) [110]. Between four and five cystein residues were labelled, over the 35 cystein residues BSA and HSA contain. A luminescent Re(I) complex bearing an epoxide-functionalized bidentate ligand (**41**, Figure 15) was used to label various cystein mutants of cytochrome P450 BM3 heme domain [111]. Interestingly, the 5,6-epoxy-5,6-dihydro-[1,10] phenanthroline bidentate ligand could be coordinated to other metal centers (e.g. Ir^{III}, Ru^{II}, Os^{II}).

Recently, Lo and co-workers published a Re(I) tricarbonyl complex functionalized with a dibenzocyclooctyne (DIBO) (compound **42**, Figure 15) for labelling azide-modified biomolecules using copper-free azide-alkyne cycloaddition [30]. They could modify BSA and HSA with an azide function at *N*-terminal position, and then label the azide-modified proteins with the DIBO- appended rhenium complex. The same group also designed tetrazine-appended Re(I) tricarbonyl complexes (**43,44**, Figure 15) for labelling bicyclo[6.1.0]nonyne (BCN)-modified proteins through an inverse electron-demand Diels-Alder reaction between the BCN and the tetrazine [112]. They could label efficiently BCN-modified apo-transferrin (aTf) as well as BSA and HSA. Interestingly, upon excitation of the ^{3}MLCT of the Re complex (*ca*. 371-390 nm), the luminescence of the tetrazine-appended Re(I) complexes (at *ca.* 515-546 nm) was quenched due to energy transfer to the non-emissive tetrazine transition at ca. 540 nm (Figure 16, molecules **44**). Upon reaction with dienophile-labelled proteins, the tetrazine moiety led to a non-quenching moiety and the luminescence was restored. This is thus an outstanding example of phosphorogenic Re(I) complexes.

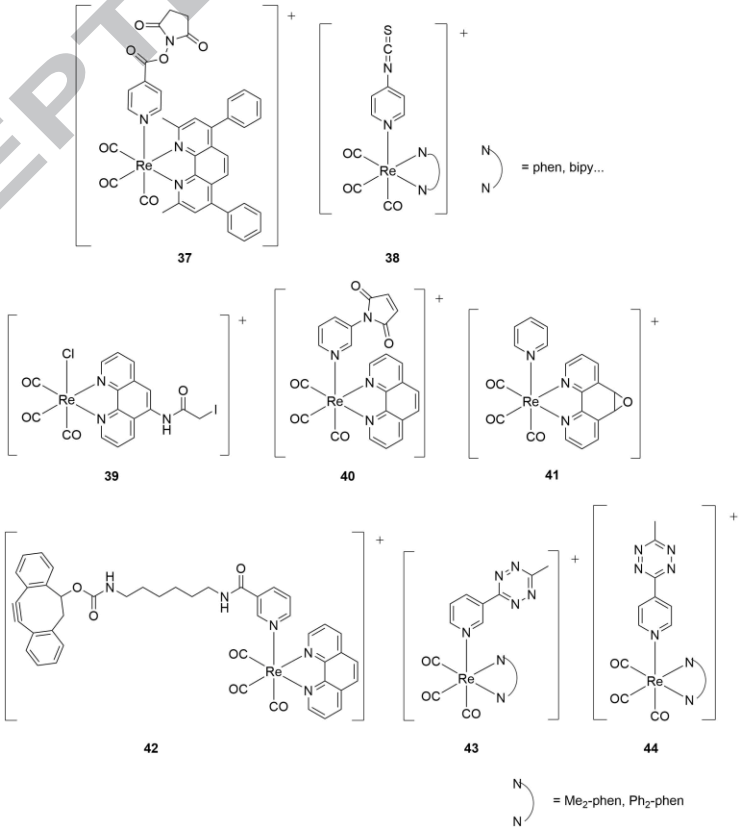


Figure 15. Examples of luminescent Re(I) tricarbonyl complexes for amine (1st line), thiol (2nd line) and bio-orthogonal (3rd line) protein labelling [30,52,107–112].

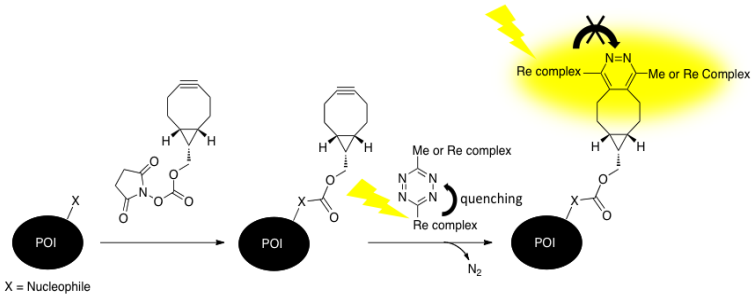


Figure 16. Labelling of a protein of interest (POI) by a Re appended tetrazine complex adapted from ref [112]

As mentioned earlier, the area of the IR bands corresponding to the coordinated CO can be used to reliably quantify the complex. Indeed, the area under the A_1-band has been shown to be weakly dependent on the environment [113] and efficient for quantification in biological samples where the polarity of the environment can change to a large extent [14]. On the other hand, the energy position of the bands, including the E-bands, can vary depending on the environment [114]. More generally, vibrational spectroscopies may be used to probe the structure and dynamics of biomolecules [115–118]. The position, width, lifetimes of signal of IR probes may for instance give information on the local conformation and dynamics of a protein or on the local electric field near a catalytic site. Although other IR probes (nitrile, azide, etc.) can be used, $Re(CO)_3$ display more intense signals [119], thus allowing a better sensitivity. *In vitro* labelling of proteins with $Re(CO)_3$ complexes may thus be of use not only for further vibrational imaging, but also to study intrinsic properties of the protein. Towards this goal, non-luminescent $Re(CO)_3$ complexes have been grafted onto proteins as for luminescent $Re(CO)_3$. Amine-specific (compounds **45, 46, 47, 48**, Figure 17) and thiol-specific (**49**, Figure 17) groups have been appended to the cyclopentadienyl rhenium tricarbonyl complex. NHS-activated esters **45, 46, 47** and pyrilium ion **48** were synthesized and used to label BSA, for instance [120,121]. Compound **49** (Figure 17) bears a thiol-reactive group frequently used for nitroxide spin labelling in EPR (Electron Paramagnetic Resonance) [119]. Upon reaction with a cystein side chain, a nucleophilic substitution occurs leading to the removal of the methanethiosulfonate group and the formation of a disulfide bond between the label and the protein. This complex (**49**) was used to label cystein mutants of ubiquitin (K6C and K63C) and α-synuclein (V71C). The authors used 2D IR spectroscopy to measure lifetime and frequency of symmetric stretching band in various solvents and when bound to the proteins. They could relate these parameters to solvation and electrostatic field, which gave insights into the structure and dynamics of the proteins.

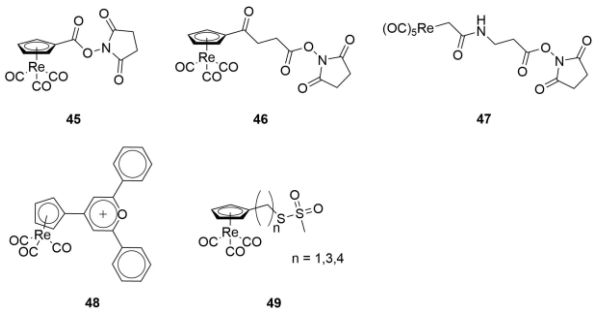


Figure 17. Examples of non luminescent Re(I) carbonyl complexes for amine and thiol protein labelling for IR studies.

The above mentioned strategies involve the functionalization of the ligand of the Re(I) complexes to make it reactive toward the protein of interest. An interesting alternative takes advantage of the reactivity of aqua Re(I) tricarbonyl complexes with amino acids side chains such as histidine imidazole (Figure 18) [86,122–125]. A luminescent aqua complex $[Re(CO)_3(phen)(H_2O)]^+$ (phen = 1,10-phenanthroline, compound **50**) and its derivate $[Re(CO)_3(dmp)(H_2O)]+$ (dmp =4,7-dimethyl-1,10-phenanthroline) were shown to label His residues of azurin from Pseudomonas aeruginosa and mutants by substitution of the aqua ligand by the imidazole ring of histidine (see **51**, Figure 18) [123]. Photoexcitation of $[Re(CO)_3(N^N)(His)]$ in the labelled azurins led to the oxidation of the copper center of the protein. Interestingly, the carbonyl ligands were used in these studies to sense the environment of the complex by Time-Resolved Infrared (TRIR) spectroscopy, thus enabling the authors to study structural changes of the protein, as well as electron transfer processes. It may thus be considered as an example of the use of the multimodality of Re(I) tricarbonyl complexes.

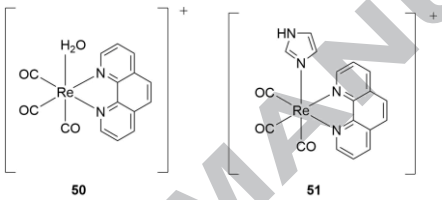


Figure 18. An aqua complex for Re(I)-modification of azurin and its resulting imidazole adduct [123].

3.2. Non covalent labelling of proteins

The protein labelling methods described above, although useful for proof of concept and for *in vitro* studies of purified proteins, are difficult to apply for the selective labelling of proteins in a complex cell environment. To the best of our knowledge, fluorescence cell imaging of proteins with Re(I) tricarbonyl has only been performed using non-covalent methods, *i.e.* by labelling a specific ligand of the protein of interest with a luminescent Re(I) tricarbonyl complex. For instance, biotinylated complexes **4** and **5** were designed as cross-linkers for avidin. Similarly, Re(I) tricarbonyl complexes were appended to estrogen derivatives to target estrogen receptors [9,20] or to indoles to target indole-binding proteins [20]. Doyle et al. conjugated luminescent Re(I) bisquinoline complexes to folic acid and to vitamin B12 to target folate receptor and cubilin receptor, respectively [50,97]. We also mentioned in this review the conjugation of Re(I) complexes to bombesin to target GPR receptor. The inertness of $Re(CO)_X$ complexes and the recent developments for the *in cells* specific labelling of proteins may open the way to the use of such complexes for live bio imaging of proteins.

Conclusion

Owing to their unique photophysical properties, Re(I) metal carbonyl complexes have been extensively used in biological contexts as probes, ^{99m}Tc surrogates, drugs and as photosensitizers for photodynamic therapies. In this review we focused on their application as probes for bio-imaging, and in particular for the imaging of peptides and proteins. Several strategies aiming at labelling peptides and proteins with Re complexes have been highlighted. These strategies have enabled the study of these biomolecules at different scales by several bio-imaging techniques involving UV-vis or infrared light. Although tuning the photophysical properties of the $(L)(X)Re(CO)_x$ complexes can be achieved by modifying the ligands (L and X), no rational have clearly emerged so far. Examples of probes possessing fully biocompatible properties enabling live imaging are still lacking and this constitutes a limitation for the use of these

complexes. However, the inertness of some $Re(CO)_X$ complexes, the ease of their synthesis and functionalization may offer the opportunity to develop suitable probe for live imaging by IR or fluorescence.

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Keywords: Re carbonyl complexes • photophysical properties • bio-imaging • peptide and protein labelling• drugs

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Highlights

- The photophysical properties of $Re(CO)_x$ derivatives used for biological application are the main topic
- The biological application of $Re(CO)_x$ derivatives are reviewed
- Photoresponsive $Re(CO)_x$ complexes used as CORMs and for PDT are described
- Strategies for labelling of peptides and proteins with $Re(CO)_x$ are presented