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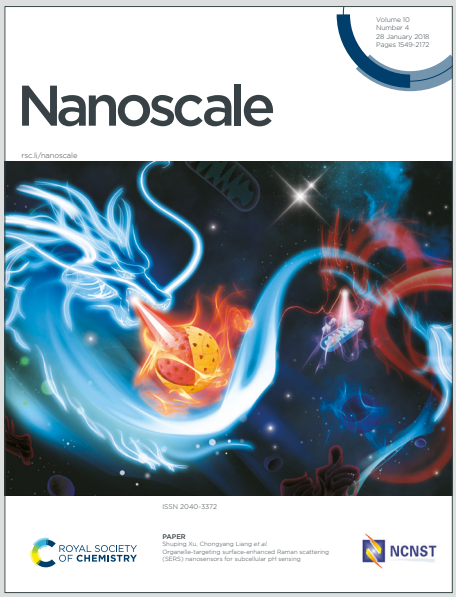

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ARTICLE

LEAFY protein crystals with a honeycomb structure as platform for selective preparation of outstanding stable bio-hybrid materials

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Well-organized protein assemblies offer many properties that justify their use for the design of innovative bionanomaterials. Here, crystals of the oligomerization domain of the LEAFY protein from Ginkgo biloba, organized in a honeycomb architecture were used as a modular platform for the selective grafting of a ruthenium-based complex. The resulting bio-hybrid crystalline material was fully characterized by UV-visible and Raman spectroscopies as well as by mass spectrometry and LC-MS after selective enzymatic digestion. Interestingly, insertion of complexes within the tubular structure affords a impressive increase in stability of the crystals eluding the use of stabilizing cross-linking strategies .

Introduction

The field of nanotechnologies has emerged with the prophetic lecture given by R. Feynman to the American Physics Society in 1959: "There's plenty of room at the bottom" in which was raised the possibility of manipulating matter at the atomic and molecular level in order to form new materials.[1] Sixty years later, thanks to the development of nanotechnologies (design, handling, characterization and use), it was established that nanometric materials allow often access to different and unexpected properties of the micro or macroscopic material, opening the door to potential applications to new domains.[2]

Some biomacromolecules, such as DNA and proteins, have the potential to self-assemble into sophisticated highly ordered nanostructures with various topologies providing specific functions. As the spatial arrangement of proteins is a prerequisite for their specific biological functions, advances in both chemistry and biotechnologies were necessary for the conception of manmade nanomaterials derived from proteins with controlled architecture (i.e. cages, fibers, tubes, multiscale layers and crystals).[3-8] Such well-organized protein assemblies have properties which render them useful for the design of innovative bionanomaterials: i) stability and solubility under biological conditions, ii) a hierarchical organization of individual building blocks into larger scale structures, iii) a confined and chiral environment, iv) the availability of residues offering many functionalizable sites and v) the possibility to modulate the protein building block by genetic manipulations.

Taking advantage of both the versatility of synthetic materials and the properties of controlled assembly of biomolecules, several bio-hybrid systems combining either nanoparticles, organic synthetic molecules or metal complexes on or within a protein assembly were reported for applications in various domains such as biocatalysis, nanodevices, medical imaging, drug delivery, diagnosis and therapy.[9-25] Yet, even if it is a field in full expansion, the variety and the number of proteins used remains poorly exploited despite their high potential. This is particularly true for protein crystals that appear to be promising candidates for the design of functional materials since they offer the following advantageous properties: i) a highly ordered monomers arrangements that form a variety of porous structures, ii) a confined and chiral interior space with solvent-filled channels that allows the diffusion of various small functional molecules, iii) a high catalyst loading capacity can be expected due to high molecular concentrations and finally iv) the possibility to design improved biocatalysts guided by the atomic resolution details of crystal structures. However, despite the impressive craze in the development of artificial enzymes, little is done in such specific area.[26]

In this paper, crystals of the LEAFY protein, which gather serendipitously all the characteristics necessary to produce bio-hybrid materials were selected: A regular honeycomb crystal architecture forming long nanotubes (5 nm x0.5mm) that allow for the diffusion of chemicals and a free protein C-terminal tail directed in the solvent channel that can be used as modular platform to graft molecules of interest. As a proof of concept, we report the selective bio-conjugation of a ruthenium-based complex grafted within the regular nanotubes in the crystals of LEAFY. The bio-hybrid system was fully characterized by spectroscopic methods and LC-MS analyses. Particularly, it was observed that the introduction of such complexes results in the formation of a highly stable (chemically and

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over time) biohybrid material that does not need to be further stabilized by cross-linking strategies.

Results and Discussion

LEAFY protein crystal as modular template for the preparation of bio-hybrid materials

Recently, the crystal structure of the Sterile Alpha Motif (SAM) domain of the LEAFY protein from Ginkgo biloba (GbLFY-SAM) was solved.[27] The LEAFY protein is a transcription factor involved in plant meristem division and differentiation.[28] Each monomer of the GbLFY-SAM (LEAFY WT) (111 amino-acid residues without any cysteine) interacts with another monomer *via* head-to-tail interaction resulting in a helix-shaped oligomer (Fig. 1a).[27] Each helical oligomer interlocks with six others leading to a honeycomb architecture with tight interaction between the edge of each helix and the groove of its six neighbors (Fig. 1a). Most of the interactions leading to the formation of the primary helix and the honeycomb architecture involve hydrogen bonds and electrostatic interactions. This results in the formation of a crystalline honeycomb structure showing parallel nanotubes with interesting features. LEAFY crystals display specific features that make them eligible objects for biotechnology applications and for industrial use: i) The crystals of long needle morphology (up to 0.5*0.07*0.07 mm^3), which can be further reinforced and colored by Ru-complexes (in this paper), are easy to handle; [29] ii) The protein shape, the crystal symmetry, the oligomer assembly and the high solvent content (57%) forms a network of long internal nanotubes (~5 nm) along the crystal C-axis that can serve as a nano-fluidic system to channel many small chemicals within the crystals; iii) A single crystal concentrates more than 45 000 billions of monomers allowing a very high loading in complex, which constitutes certainly a major asset for the design of bio-hybrid systems; iv) a C-ter disordered extremity constituted by 38 residues (RAEKRRLDELERK$_{84}$KLDLFVDVDGKRKADENALDTLSQA) is floating inside the crystal channels, and can be used as a modular platform for the grafting of various species to achieve fine chemistry. Only the first twelve residues of the C-terminal sequence are observable in the X-ray crystal structure, probably due to a lack of regular arrangement of a major part of the side chains or to an important flexibility and thermal disorder. However, the integrity of the chain in the crystal was verified by MS analysis (Fig. S1, ESI).

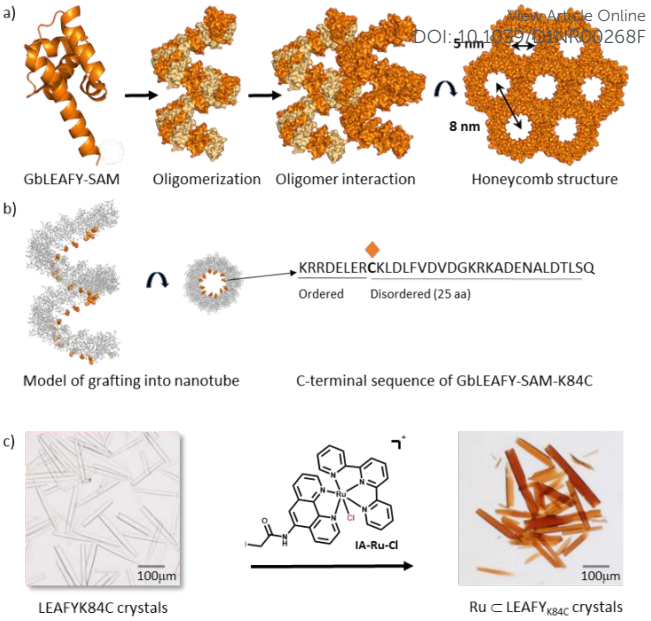

Fig. 1. (a) Self-assembly of LEAFY WT into a honeycomb architecture leading to the formation of a tubular structure. The diameter of each tube as well as the distance between the centre of two tubes are indicated ; (b) Synthetic view of the honeycomb structure and of the targeted complex localization ; (c) Grafting process of IA-Ru-Cl into LEAFY$_{K84C}$ crystals.

Preparation of the bio-hybrid material

For a selective functionalization of this material, it was decided to introduce a single cysteine residue per monomer as a unique binding site. This was achieved by mutation of the lysine 84, first residue of the disordered side chain closed to the wall of the tube (see above) (LEAFY$_{K84C}$) without noticeable modification of the crystals characteristics. It was also anticipated that introduction of a relatively large charged molecule with aromatic ligands could potentially increase the rigidity and/or the order in the internal structure thanks to additional interactions (i.e. π-stacking, electrostatic interactions). Using a crystallization buffer containing Tris-HCl (12.5 mM, pH7.2), ammonium sulfate (550 mM) and Tris(2-carboxyethyl)phosphine (1mM), conditions were optimized to get a good reproducibility in terms of crystals size and number in each crystallization well. Protein amount in crystals issued from every well of crystallisation was then determined to be 112.10^{-12} mol by electrophoresis gel using LEAFY WT as standard. In order to assess the feasibility of a controlled grafting inside the tubes at selected sites, we used a $[Ru(tpy)(phen)Cl]^{2+}$-derivative (with tpy, terpyridine and phen, 1-10-phenanthroline) as polypyridyl ruthenium complex mainly for its high absorption properties providing a typical spectroscopic footprint useful for the characterization of the material and for its potential catalytic activity.[30-37]

Overnight soaking crystals with an excess (14 equiv./cysteine on the basis of electrophoresis determination) of $[(IA\text{-}phen)(tpy)Ru\text{-}Cl]^+$ complex (IA-Ru-Cl) (Fig. 1c) (obtained from $RuCl_3$ in 3 steps (Fig. S2, ESI†) and functionalized with a iodoacetamide function (IA) known to be selective of cysteine residues[38,39] results in a permanent and deep coloration of the crystals. The persistence of this coloration after intensive washing strongly suggests a covalent binding of the

complex in the crystal (Fig. 1c). This assumption was confirmed by a control experiment with [(tpy)(5-NH_2-phen)RuCl]$^+$ (NH_2-Ru-Cl with 5-NH_2-phen, 5-amino-1,10-phenanthroline) complex. This complex which does not near any anchoring function is indeed entirely eliminated during washing resulting in colorless crystals. This result also suggests that substitution of the chloro ligand by suitable ruthenium ligands, such as the amino group of lysine and arginine or imidazole group of histidine, does not occur in such conditions. Unfortunately, X-Ray diffraction of the resulting material did not allow to observe any ruthenium complex within the structure (Table S1, ESI†). Indeed, no positive residual electron density that could correspond either to Cys84 or to the complex was able to be observed in the Fo-Fc electron density map. The last residues of the structure are poorly defined in the 2FoFc electron density map. These observations suggest that the complex is either bound to a flexible part of the protein preventing its observation in the electron density map or is released by radiation damage after a possible X-ray induced cleavage of the C-S bond.[40,41] X-ray diffraction structure would have provided important information about the selectivity of the grafting and the localization of the complex. Hence, without such information, alternative approaches to characterize the bio-hybrid material were used.

Selectivity and efficiency of the grafting

We first investigated the efficiency and the selectivity of the binding. $LEAFY_{K84C}$ crystals were soaked in the presence of different concentrations of IA-Ru-Cl and the amount of grafted ruthenium was quantified by inductively coupled plasma mass spectrometry (ICP-MS) after different incubation times (Fig. 2). It was observed that with the initial conditions (14 equiv. of complex, overnight) about a two-fold excess of ruthenium complex was anchored indicating the existence of an additional non-specific binding. This was confirmed by the fact that about 1 equivalent of complex per monomer was grafted in LEAFY WT soaked in the same condition, since no cysteine residue is present in this case (Fig. S3, ESI†).

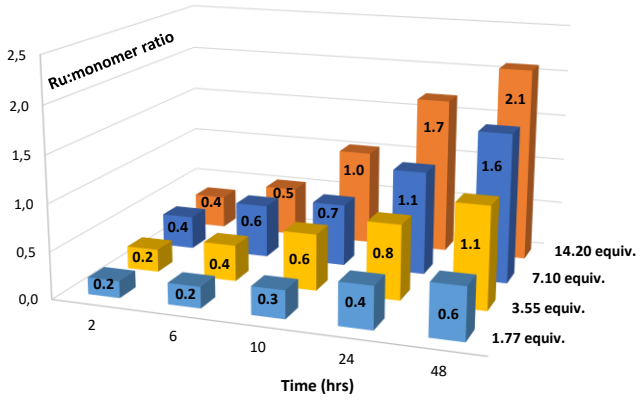

Fig. 2. Ratio of grafted complex (determined by ICP-MS) per monomer of $LEAFY_{K84C}$ unit according to the number of IA-Ru-Cl complex equivalents added and the soaking time.

However, liquid chromatography mass spectrometry analysis (LC-MS) of the $LEAFY_{K84C}$ bio-hybrid material after crystal dissolution in the presence of 5% formic acid and sonication for 1h, showed a unique peptide modified by a single molecule of the complex (Fig. S4, ESI†). Its isotopic pattern shows three peaks at m/z 13355.70, 13374.69 and 13396.70 corresponding to the grafted complex where the chloro ligand was lost (named Ru ⊂ $LEAFY_{K84C}$) or replaced by either a water molecule (Ru-OH_2 ⊂ $LEAFY_{K84C}$) or acetonitrile (Ru-CH_3CN ⊂ $LEAFY_{K84C}$) coming from the elution solvent respectively. Moreover, the absence of any trace of non-grafted protein allows us to assess the effectiveness of the grafting. Unexpectedly no peak corresponding to a peptide bearing two grafted complexes could be detected. The same experiment was carried out with the WT hybrid, however, the obtained spectrum was identical to that of the protein alone (data not shown). These results indicates that the interaction of the non-specific complex does not resist the acidic conditions required to solubilize both hybrids. Finally, when the reactivity toward IA-Ru-Cl of LEAFY WT and $LEAFY_{K84C}$ both in solution were compared, only the grafting of the latter bearing a cysteine residue was achieved as observed by LC-MS (Fig. S4 and S5, ESI†). This result unambiguously shows the high selectivity of the grafting once the mutant protein is in solution contrary to what is observed in the crystals. Unfortunately, despite more than 900 conditions tested (at the htxlab platform at the European Molecular Biology Laboratory-Grenoble), we were unable to obtain crystals of the functionalized protein after modification in the liquid state for XRD analysis.

Competitive reactions with other amino-acids, mainly lysine residues, are known. However, this inconvenient can be avoided using chloroacetamide derivative (ClA).[42] However, albeit the kinetic proved to be slower as expected, a persistent coloration was also observed when ClA-Ru-Cl was soaked with LEAFY WT, in which no cysteine is present (Fig. S6, ESI†). This result indicates that the specific grafting of a complex on the unique cysteine residue of $LEAFY_{K84C}$ is unmanageable, since part of concomitant non-specific binding always occurs. Nevertheless, this output provides important information concerning the type of grafting of the second complex within $LEAFY_{K84C}$. Indeed, we have shown that strength electrostatic interactions alone are not sufficient to retain the complex within the crystal since NH_2-Ru-Cl is eliminated during washing. At this stage of the study, two hypotheses can be proposed. First of all, alkylation of another amino acid (i.e. histidine, lysine, methionine, aspartate or glutamate) cannot be entirely ruled out as it is known that the iodoacetamide function, albeit selective of cysteine, is not specific for this residue.[43-45] However, in such a case, the interaction would be so fragile that it would not withstand the acidic conditions needed to solubilize the crystal. Alternatively, it cannot to be excluded that grafting of the first complex allows the binding of the second via, for example, $\pi-\pi$ interactions between the two complexes.

Enzymatic digestion and LC-MS analysis

In order to further characterize the hybrid, enzymatic digestion was then investigated using Arg-C, an endoprotease that hydrolyzes peptide bonds at the carboxyl side of arginyl residues. ArgC digestion of the bio-hybrid protein showed that only the cysteine-containing peptide CKLDLFVDVDGKR was modified. Unexpectedly, LC-MS analysis of this modified peptide reveals two distinctive peaks at different retention times but with the same m/z of 424.18 (z=5) and similar MS/MS fragmentation (Fig. S7, ESI†). This result is consistent with the grafting of the two stereoisomers of the complex within the protein. MS/MS of this peptide unambiguously confirmed the

selective binding of the complex to the unique cysteine residue, as depicted in Figure 3. Indeed, while doubly-charged fragments b(2) and b(3) indicate the presence of the Ru(II) complex, fragment y(12) is composed of all the amino acid residues with the exception of both the cysteine and the complex. A similar spectrum was obtained with the soluble modified protein.

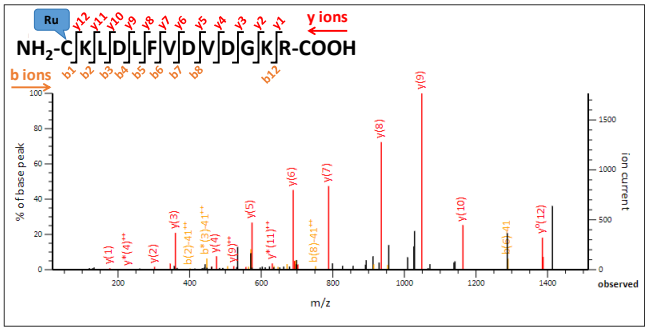

Fig. 3. MS/MS spectrum of the peptide containing the complex after ArgC digestion of Ru-OH_2 ⊂ $LEAFY_{K84C}$ crystals identical to that obtained by ArgC digestion of the soluble modified protein.

In cristallo UV-visible and Raman studies

In order to verify the integrity of the internalized complexes and to obtain other important information concerning their mode of interaction with the protein, the resulting bio-hybrid material issued from $LEAFY_{K84C}$ was then characterized by UV-vis[46] and Raman[47] spectroscopies. UV-vis absorption measurements of IA-Ru-Cl alone in acetone solution showed the expected spectrum with the typical broad band at 510 nm corresponding to a Metal-to-Ligand-Charge-Transfer (MLCT) (Fig. 4). Interestingly, this band was blue-shifted to higher energy when the complex is grafted in the crystal (from 510 to 495 nm). This could correspond to the substitution of the chloro ligand by a water molecule during the grafting process, resulting in the formation of Ru-OH_2 ⊂ $LEAFY_{K84C}$. Indeed, we showed that addition of 95% of water in a DMSO solution of IA-Ru-Cl presents a spectrum similar to that obtained with $[(phen)(tpy)Ru\text{-}OH_2]^{2+}$.[48] It is worth noting that such a substitution usually requires acidic conditions or the presence of silver salt to assist in departure of the chloride.[48-52]. Moreover, the presence of the additional ruthenium complex in the crystal could also explain the observed broadening of the band.

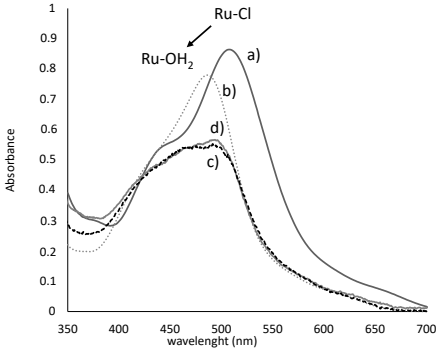

Fig. 4. Absorption spectra (a) of IA-Ru-Cl in acetone (80µM) ; (b) of IA-Ru-OH_2 obtained from IA-Ru-Cl in a 95:5 H_2O:DMSO mixture (80µM) ; (c) of $LEAFY_{K84C}$ crystals grafted with IA-Ru-Cl in a 1:2 acetone:H_2O mixture ; (d) of $LEAFY_{K84C}$ crystals grafted with IA-Ru-OH_2 previously obtained from IA-Ru-Cl in a 98:2 H_2O:DMSO mixture.

Then, as described in the method section[47,53] Raman spectroscopy measurements were performed on a powder of NH_2-Ru-Cl, on a crystal of native $LEAFY_{K84C}$, as well as on the biomaterials Ru-$^{16}OH_2$ ⊂ $LEAFY_{K84C}$ and Ru-$^{18}OH_2$ ⊂ $LEAFY_{K84C}$ formed in a solution containing $H_2{}^{16}O$ and $H_2{}^{18}O$ respectively. When using an excitation source near the maximum absorption of the MLCT (514 nm) the fluorescence re-emitted by samples were observed to be unmanageable and prevented measuring Raman data at the resonance. Hence, a near-infrared excitation source (785 nm, 50 mW) was chosen to reduce this massive fluorescence.

Overall inspection of the raw data showed that Raman spectrum of the NH_2-Ru-Cl powder (Fig. S8-A, ESI†) displays relatively strong peaks probably due to a pre-resonance effect, since the 785nm excitation line is still situated in the far low energy tail of the absorption spectrum of the complex. The Raman spectrum of native $LEAFY_{K84C}$ crystal (Fig. S8-C, ESI†) exhibits very weak peaks severely obscured by strong fluorescence, while contrarily the spectrum of Ru-$^{16}OH_2$ ⊂ $LEAFY_{K84C}$ crystal (Fig. S8-B, ESI†) reveals a more balanced intensity ratio between Raman peaks and fluorescence.

Interestingly, the comparison of these three spectra allowed to infer that, for the Ru-OH_2 ⊂ $LEAFY_{K84C}$ crystal, the enhancement of the Raman signal from vibrations of protein $LEAFY_{K84C}$ only is likely induced by its grafted pre-resonant complex. This observation allowed to propose the use of exogenous complexes as a possible method to enhance Raman signals in different proteins crystals. Indeed numerous native proteins crystals exhibit weak Raman signals often hidden by luminescence/fluorescence. The whole Raman spectra of these hybrids crystals would reveal exalted peaks, but this assumes being capable of discriminating Raman-bands of the proteins from those of the complexes that could be convoluted.

The Figure S9 (ESI†) shows the Raman spectra after fluorescence background corrections and a qualitative rescaling based on strongest peaks to make the comparison possible. Unexpectedly, the corrected spectrum of native $LEAFY_{K84C}$ crystal (Fig. S9-C, ESI†) appears to be qualitatively exploitable. This spectrum is contaminated by a huge peak of the vibration ν(SO_4) of ammonium sulfate used for crystallization, while this band appears to be strongly reduced for the hybrid Ru-OH_2 ⊂ $LEAFY_{K84C}$ crystals which were back-soaked (Fig. S9-B, ESI†). In both spectra, the vibrational peaks at 1650 and 1340 nm are obviously attributed to the Amides bands I and III respectively, the values of which confirm that $LEAFY_{K84C}$ secondary structure is indeed dominated by α-helices.

The whole Raman spectra (300-1700 cm^{-1}) can be roughly subdivided in 3 regions: (i) A low frequencies sector that comprises vibrations of metal-ligand bonds (300-500 cm^{-1}, Fig. 5-I), (ii) an intermediate sector that contains vibrations of single-bonds between C, N, 0, S atoms (500-1000 cm^{-1}, Fig. 5-II) and (iii) the high frequencies sector that covers the heterocycle bounds vibrations of pyridine derivatives (1000-1700 cm^{-1}, Fig. 5-III and IV).

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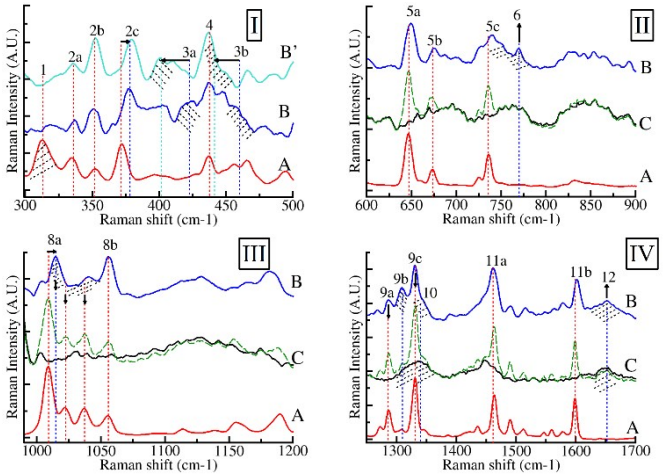

Fig. 5. Raman spectra of pure NH_2-Ru-Cl powder (A in red), the protein native $LEAFY_{K84C}$ (C in black) and the hybrid Ru-$^{16}OH_2 \subset LEAFY_{K84C}$ (B in blue) and Ru-$^{18}OH_2 \subset LEAFY_{K84C}$ (B' in cyan) crystals. Spectral region 300-1700 cm^{-1} is subdivided in 3 sectors: the low frequencies region (I), the intermediate region (II), and the high frequencies region (III and IV). The main vibration peaks are labelled from 1 to 12 and are attributed in table S2. The green dashed curve is the simple addition of NH_2-Ru-Cl and native $LEAFY_{K84C}$ Raman spectra, which serves as a qualitative guide for the eyes for the comparison with Ru-$^{16}OH_2 \subset LEAFY_{K84C}$.

The possible peak assignments, listed in table S2 (ESI†), were based on experimental and computational spectroscopic studies on ruthenium polypyridinyl complexes, and pyridine derivatives either free or metal-complexed.[46, 53-55] In the low-frequency zone, we first observed a vibration mode at 312 cm^{-1} in the pure complex spectrum (peak-1 in Fig. 5-I-A) that is strictly absent in those of the hybrid crystals (Fig. 5-I-B and B'). On the contrary, we observed broad peaks 3ab in the sector 410-430cm^{-1} exclusively for the hybrid crystals, which undergo a shift of 20cm^{-1} upon $H_2{}^{16}O/H_2{}^{18}0$ isotopic substitution (Fig. 5-I-B and B'). Therefore, as assumed in the above UV-visible studies, this result confirms the substitution of the chloro ligand by a water molecule during the grafting process, since peak 1 is attributed to the vibration of Ru-Cl bond[56] while peak 3 to Ru-OH_2.[57] Bands 2abc are assigned to stretching vibrations of the remaining Ru-N metal-ligand bonds, and peak 2c that shifts by 5 cm^{-1} upon complex-protein hybridation is attributed to the phenanthroline ligand.[58] Most importantly, in the intermediate zone (Fig. 5-II), apart bands 5ab that are attributed to collective deformations motions of heterocycles, we clearly identified a broad region 750-780 cm^{-1} with the dominant peak 6 pointing at 770 cm^{-1} in the hybrid spectrum (Fig. 5-II-B). This peak, which is rigorously absent in the both spectra of the pure complex and the native protein crystals (Fig. 5-II-A and C), corresponds to the stretching vibration of a newly formed "C-S" bond,[59-61] and constitutes the spectroscopic evidence for the selective covalent grafting of the complex to the cysteine residue. The high frequencies zone (Fig. 5-III and IV) contains the strongest "pre-resonant" Raman bands typical of stretching vibration of bonds internal to phenanthroline and terpyridine heterocycles. We observed that stretching modes intensities of single and hybrid bonds (peaks 8 and 9 at around 1000 and 1300 cm^{-1} respectively which are the prominent bands in the free complex-Fig. S9) are strongly reduced in the complex-protein hybrid as compare to the intensity of the double bonds (peaks 11ab, at 1463 and 1598 cm^{-1} respectively) that are less affected by the complexation. We speculate that the strong reduction of pre-resonance for the hybrids could stem from either a π-π interaction between an heterocyclic ligand of the anchored complex grafted to protein and another one of the second complex, or a π-π interaction between the non-specific complex and an aromatic amino acid.[62]

Location of the non-specific grafted complex

Undeniably, it is clear that the cysteine is necessary for the grafting of a first complex as observed on $LEAFY_{K84C}$ either in the crystal or in solution. Furthermore, since the WT protein in solution was unable to bind any complex (Fig. S5, ESI†), these results show that both crystalline structures allows the internalization of a non-specific complex and implies particularly weak interactions that does not resist the acidic conditions required for the dissolution of the hybrid crystals. It has been reported that the reactivity of amino acids toward alkylating reagent such as iodoacedamide is ranked as follow Cysteine (SH)> N-terminus (NH_2)>Glutamic acid (CO_2H) >C-terminus=lysine(NH_2)>aspartic acid (CO_2H) >tyrosine (OH)>histidine (imidazole).[63] A closer look at the LEAFY structure reveals that, in addition to all the residues that compose the C-ter tail oriented in the solvent channel, both the amino group of the N-terminus glycine and carboxylate groups of some glutamic acids seem accessible to the complex (Fig. S10, ESI†); the unique histidine is located in the wall of the tube and therefore inaccessible to the complex. However as the non-specific grafted complex is released during acidic dissolution of both Ru ⊂ LEAFY K84C and Ru ⊂ LEAFY WT hybrids, N-alkylation of the N-terminus glycine, lysine or histidine residues is excluded since it would lead to the formation of alkylated amines reputed to be particularly stable (Fig. S11, ESI†). On the other hand, without being able to totally exclude the intervention of a π–π stacking type interaction with aromatic amino acids such as the phenylalanine (F89) located in the side chain, O-alkylation of a glutamic acid located either in the side chain (E80, E82, E100) or at the inner surface of the tube can be privileged. In that case, the alkylation leads to the formation of an ester, function known to be hydrolysable in aqueous acid conditions (Fig. S11, ESI†).

Stability of the bio-hybrid material

Finally, it was observed that introducing the complex results in an notable increase in stability of both the crystals as well as the grafted complex. In order to determine whether the observed crystal stability is due to the introduction of the cysteine or of the complex, a comparative stability study was carried out on LEAFY WT, Ru ⊂ LEAFY WT and Ru-$OH_2 \subset LEAFY_{K84C}$ (bearing 0, 1 (Fig. S3, ESI†) and 2 complexes per monomer respectively), in acidic conditions. It was observed that, while a few minute suffice to dissolve $LEAFY_{K84C}$ in a 0.05% formic acid solution, 0.5% is required to dissolve Ru ⊂ LEAFY WT, and a much higher concentration (5%) followed by at least 1h of sonication is needed to solubilize Ru-$OH_2 \subset LEAFY_{K84C}$. (Fig. S12, ESI†). These results clearly show the correlation between stability and the number of grafted ruthenium complexes. The impressive stability of the modified Ru ⊂ LEAFY WT and Ru-$OH_2 \subset LEAFY_{K84C}$ bio-hybrid materials was also observed in various organic solvents such as

acetone, acetonitrile, dimethylformamide and dimethylsulfoxide (DMSO) (Fig. S12, ESI†). Even irradiation of the bio-hybrid material for 2 days with a blue led light (455 nm), or during several months with daylight, in either water, acetonitrile or DMSO, did not cause any change in the appearance or color of the crystal.[64-66] In comparison, blue light irradiation for 48h of the complex alone in a 1:1 mixture of CH_3CN-DMSO solution resulted in the total photosubstitution of at least, the chloro ligand and/or the phenanthroline by either acetonitrile or DMSO molecules (Fig. S13, ESI†).[67] We may hypothesize that the observed stability of both the crystal and the complex is a consequence of combination of covalent and π-stacking interactions between the complexes and the protein. It is worth noting that this extremely high robustness is a property of importance for the development of stable crystalline artificial metalloenzymes, since usually protein crystals reveal a strong natural inherent fragility and thus need to be further stabilized by cross-linking strategy in order to serve in chemical applications.[68-70]

Conclusions

This research reports the full characterization of a bio-hybrid material obtained by bioconjugation of a ruthenium complex within the porous LEAFY protein crystal. This was achieved by using combined approaches mainly UV-visible and Raman spectroscopies performed directly on the crystals coupled to enzymatic digestion and subsequent LC-MS analysis of the resulting fragments. The resulting material demonstrates a noteworthy increase in stability in both organic and aqueous media without the need of further cross-linking strategies. Remarkably since the various mutations achieved on the inner side chain do not affect the honeycomb structure of the crystal,[71] this works opens an interesting path to the elaboration of solid porous bio-hybrid materials using the easy handling LEAFY protein crystal as a modular platform

Conflicts of interest

There are no conflicts to declare.

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Notes and references

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‡ **Supporting Information**. Electronic supplementary information (ESI) available: Synthesis of the complexes, overexpression, purification and crystallization of proteins as well as grafting procedures and full hybrid characterization are detailed in supporting information. Spectral data of compounds are also included.

1 R. P. Feynman *Eng. Sci*. 1960, **23**, 22.
2 S. Bayda, M. Adeel, T. Tuccinardi, M. Cordani, F. Rizzolio, *Molecules* 2020, *25*, 112
3 C. Yuan, A. Levin, W. Chen, R. Xing, Q. Zou, T. W. Herling, P. Kumar Challa, T. P. J. Knowles, X. Yan, *Angew. Chem. Int. Ed*. 2019, **58**, 18116.
4 C. Yuan, W. Ji, R. Xing, J. Li, E. Gazit, X. Yan, *Nat. Rev. Chem*. 2019, **3**, 567.
5 L. Wang, C. Gong, X. Yuan, G. Wei, *Nanomaterials*, 2019, **9**, 285.
6 S. L. Kuan, F. R. G. Bergamini, T. Weil, *Chem*. *Soc*. *Rev*. 2018, **47**, 9069.
7 Q. Luo, C. Hou, Y. Bai, R. Wang, J. Liu, *Chem*. *Rev*. 2016, **116**, 13571.
8 B. J. G. E. Pieters, M. B. van Eldijk, R. J. M. Nolte, J. Mecinovic, *Chem*. *Soc*. *Rev*. 2016, **45**, 24.
9 S. Abe, B. Maity, T. Ueno, *Chem*. *Commun*. 2016, **52**, 6496.
10 J. G. Heddle, S. Chakraborti, K. Iwasaki, *Curr*. *Opin*. *Struct*. *Biol*. 2017, **43**, 148.
11 Q. Luo, C. Hou, R. Wang, J. Liu, *Chem*. *Rev*. 2016, **116**, 13571.
12 T. Matsuo, S. Hirota, *Bioorg. Med. Chem*. 2014, **22**, 5638.
13 N. J. M. Sanghamitra, T. Ueno, *Chem*. *Commun*. 2013, **49**, 4114.
14 N. Yokoi, Y. Miura, C.-Y. Huang, N. Takatani, H. Inaba, T. Koshiyama, S. Kanamaru, F. Arisaka, Y. Watanabe, S. Kitagawa, T. Ueno, *Chem*. *Commun*. 2011, **47**, 2074.
15 S. Abe, J. Niemeyer, M. Abe, Y. Takezawa, T. Ueno, T. Hikage, G. Erker, Y. Watanabe, *J. Am. Chem. Soc*. 2008, **130**, 10512.
16 A. L. Margolin, M. A. Navia, *Angew. Chem. Int. Ed*. 2001, **40**, 2204.
17 H. Tabe, H. Takahashi, T. Shimoi, S. Abe, T. Ueno, Y. Yamada, *App. Catal*. *B : Environ*., 2018, **237**, 1124.
18 S. Lopez, L. Rondot, C. Leprêtre, C. Marchi-Delapierre, S. Ménage, C. Cavazza C, *J. Am. Chem. Soc*. 2017, **139** ,*49*, 17994.
19 S. Abe, B. Maity, T. Ueno, *Chem*. *Commun*. 2016, **52**, 6496.
20 S. Abe, T. Ueno, *RSC adv.* 2015, **5**, 21366.
21 N. J. M. Sanghamitra, T. Ueno, *Chem. Commun*. 2013, **49**, 4114.
22 T. Ueno, H. Tabe, Y. Tanaka, *Chem. Asian J*. 2013, **8**, 1646.
23 C. Cavazza, C. Bochot, P. Rousselot-Pailley, P. Carpentier, M. V. Cherrier, L. Martin, C. Marchi-Delapierre, J. C. Fontecilla-Camps, S. Ménage, *Nat. Chem*. 2010, **49**, 7014.
24 M. A. Kostiainen, P. Hiekkataipale, A. Laiho, V. Lemieux, J. Seitsonen, J. Ruokolainen, P. Ceci, *Nature Nanotechnology* 2013, **8**, 52
25 F. Setaro, M. Brasch, U. Hahn, M. S. T. Koay, J. J. L. M. Cornelissen, A. de la Escosura, T. Torres, *Nano Lett*. 2015, **15**, 1245
26 S Datta, L. R. Christena, Y.R.S. Rajaram et al., *Biotech*., 2013, 3, 1.
27 C. Sayou,M. H. Nanao, M. Jamin, D. Pose, E. Thevenon, L. Gregoire, G. Tichtinsky, G. Denay, F. Ott, M. Peirats Llobet, M. Schmid, R. Dumas, F. Parcy, *Nat. Commun*. 2016, **7**, 11222.
28 G. Denay, H. Chahtane, G. Tichtinsky, F. Parcy, F. *Curr. Opin. Plant Biol.* 2017, **35**, 15.
29 O. Svenssona, M. Gilskib, D. Nurizzoa, M. W. Bowler, bioRxiv 558023; doi: https://doi.org/10.1101/558023.
30 J. M. Kamdar, D. B. Grotjahn, *Molecules* 2019, **24**, 494.

31 J. D. Blakemore, R. H. Crabtree, G. W. Brudvig, *Chem. Rev*. 2015, **115**,12974.
32 D. L. Ashford, M. K. Gish, A. K. Vannucci, M. K. Brennaman, J. L. Templeton, J. M. Papanikolas, T. J. Meyer, *Chem. Rev*. 2015, **115**, 23, 13006.
33 W. Iali, P.-H. Lanoe, S. Torelli, D. Jouvenot, F. Loiseau, O. Hamelin, S. Ménage, *Angew. Chem. Int. Ed*. 2015, **54**, 8415.
34 M. D. Kärkäs, O. Verho, E. V. Johnston, B. Åkermark, *Chem. Rev*. 2014, **114**, 11863.
35 A. Paul, J. F. Hull, M. R.Norris, Z. Chen, D. H. Ess, J. J. Concepcion,T. J.Meyer *Inorg. Chem.* 2011, **50**, 1167.
36 L. Tong, R. P. Thummel, *Chem. Sci*., 2016, **7**, 659.
37 Q. Chen, Q. Zhou, T.-T. Li, R. Liu, H. Li,F. Guo, Y.-Q. Zheng, *Trans. Met. Chem.* 2019**, 44**, 349.
38 S. B. Gunnoo, A. Madder, *ChemBioChem*. 2016, *17*, 529.
39 J. M. Chalker, G. J. L. Bernardes, Y. A. Lin, B. G. Davis, *Chem. Asian J.* 2009, **4**, 630.
40 E. F. Garman, *Acta Cryst*. 2010, D66, 339.
41 P. Burmeister, *Acta Cryst*. 2000, **D56**, 328
42 M. L. Nielsen, M. Vermeulen, T. Bonaldi, J. Cox, L. Moroder, M. Mann, *Nat. Methods* 2008, **5**, 459.
43 T. Muller, D. Winter, *mol. Cell. Proteomic*, 2017, *16.7*, 1173.
44 S. Suttapitugsakul, H. Xiao, J. Smeekens, R. Wu, *Mol. Biosyst*. 2017, **13**, 2574.
45 E. S. Boja, H. M. Fales, *Anal. Chem*. 2001, **73**, 3576.
46 D. von Stetten, T. Giraud, P. Carpentier, F. Sever, M. Terrien, F. Dobias, D. H. Juers, D. Flot, C. Mueller-Dieckmann, G. A. Leonard, D. de Sanctis, A. Royant, *Acta Cryst*. 2015, **D71**, 15.
47 P. Carpentier, A. Royant, J. Ohana, D. Bourgeois, *J. Appl. Cryst*. 2007, **40**, 1113.
48 N. Kaveevivitchai, R. Zong, H.-X. Tseng, R. Chitta, R. P. Thummel, *Inorg. Chem*. 2012, **51**, 2930.
49 D. J. Wasylenko, C. Ganesamoorthy, B. D. Koivisto, M. A. Henderson, C. P. Berlinguette, *Inorg. Chem.* 2010, **49**, 2202.
50 M. D. Kärkäs, O. Verho, E. V. Johnston, B. Åkermark, *Chem. Rev*. 2014, **114**, 11863.
51 W. Chen, F. N. Rein, R. C. Rocha, *Angew. Chem. Int. Ed*. 2009, **48**, 1.
52 O. Hamelin, P. Guillo, F. Loiseau, M.-F. Boissonnet, S. Ménage, *Inorg. Chem*. 2011, **50**, 7952.
53 A. S. Denisova, E. M. Dem'yanchuk, G. L. Starova, L. A. Myund, A. A. Makarov, A. A. Simanova, *J. Mol. Struct.* 2007, **828**, 1.
54 P. K. Mallick, G. D. Danzer, D. P. Strommen, J. R. Kincaid, *J. Phys. Chem*. 1988, **92**, 5628.
55 D. P. Strommen, P. K. Mallick, G. D. Danzer, R. S. Lumpkin, J. R. Kincaid, *J. Phys. Chem*. 1990, **94**, 1357.
56 D. K. Breitinger, R. Breiter, *J. Mol. Struct*. 1995, **349**, 45.
57 A. Vergara, G. D'Errico, D. Montesarchio, G. Mangiapia, L. Paduano, A. Merlino, *Inorg. Chem*. 2013, **52**, 4157.
58 J. K. Hurst, J. Z. Zhou, Y. B. Lei, *Inorg. Chem.* 1992, **31**, 1010.
59 P. Bazylewiski, R. Divigalpitiya, G. Fanchini, *RSC. Adv.* 2017, **7**, 2964.
60 S. F. Parker, *Chem. Phys.* 2013, **424**, 75.
61 T. H. Joo, Y. H. Yim, K. Kim, M. S. Kim, *J. Phys. Chem*. 1989, **93**, 1422.
62 W. Chen, C. Turro, L. A. Friedman, J. K. Barton, N. J. Turro, *J. Phys. Chem. B* 1997, **101**, 6995
63 S. Suttapitugsakul, H. Xiao, J. Smeekens, R. Wu, *Mol Biosyst*. 2017, **21**, 2574
64 E. Baranoff, J.-P. Collin, J. Furusho, Y. Furusho, A. –C. Laemmel, J.-P. Sauvage, *Inorg. Chem*. 2002, **41**, 1215.
65 H. B. Ross, M. Boldaji, D. P. Rillema, C. B. Blandon, R. P. White, *Inorg. Chem*. 1989, **28**, 1013.
66 D. P. Rillema, C. B. Blanton, R. J. Shaver, D. C. Jackman, M. Boldaji, S. Bundy, L. A. Worl, T. J. Meyer, *Inorg. Chem*. 1992, **31**, 1600.
67 L. N. Lameijer, C.van de Griend, S. L. Hopkins, A.-G. Volbeda, S. H. C. Askes, M. A. Siegler, S. Bonnet, *J. Am. Chem. Soc*. 2019, **141**, 352.
68 E.-K. Yan, Q.-Q. Lu, C.-Y. Zhang, Y.-L. Liu, J. He, D. Chen, B. Wang, R.-B. Zhou, P. Wu, D.-C. Yin, *Sci. Rep*., 2016, **6**, 34770.
69 J. J. Roy, T.E. Abraham, *Chem. Rev*. 2004, **104**, 3705.
70 A. L. Margolin, M. A. Navia, *Angew. Chem. Int. Ed*. 2001, **40**, 2204.
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