Bioconjugation strategies to couple supramolecular exo-functionalized palladium cages to peptides for biomedical applications

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Supramolecular Pd_2L_4 cages (L = ligand) hold promise as drug delivery systems. With the idea of achieving targeted delivery of the metallacages to tumor cells, the bioconjugation of *exo*-functionalized self-assembled Pd_2L_4 cages to peptides following two different approaches is reported for the first time. The obtained bioconjugates were analyzed and identified by high-resolution mass spectrometry.

Chemotherapy is one of the main modalities of treatment for cancer patients. However, its success rate remains limited, primarily due to limited selectivity of drugs for the tumor tissue, often resulting in severe toxicity, as well as to the development of multi-drug resistance caused by the heterogeneous biology of the growing tumors.

In general, an important challenge in cancer treatment is to find a technology for targeted delivery and controlled release of drugs to eradicate tumor cells while sparing normal ones. Therefore, considerable efforts have been devoted to the development of ***drug delivery systems*** that can overcome the above mentioned issues related to anticancer drugs used in chemotherapy.[1, 2] In some cases, it was also possible to achieve a synergistic anticancer effect of different therapeutic modalities combined in one drug delivery system.[3] Within this framework, an increasing number of reports has appeared on tethering anticancer compounds to or encapsulating them in a wide range of functional molecules or nanomaterials with or without targeting groups.[4-6] Thus, lipid nano-systems, such as liposomes and micelles along with virus-inspired vectors and polymeric particles, as well as inorganic nanoparticles, have been studied to deliver bioactive compounds to the target tissues.

In this context, supramolecular chemistry offers new opportunities for improved drug delivery systems, its principal aim being to create nanoscale structures while exerting control over their size and shape, and to emulate biological systems with synthetic ones.[7]

Interestingly, coordination-driven self-assembly utilizes the spontaneous formation of metal-ligand bonds in solution to drive mixtures of molecular building blocks to single, unique 2D metallocycles or 3D metallacages based on the directionality of the precursors used. The supramolecular coordination complexes (SCCs) obtained via this process are characterized by well-

defined internal cavities and relatively facile pre- or post-self-assembly functionalization.[8] These properties augment the modularity of the directional bonding design strategy to provide structures with unprecedented fine-tuning possibilities, spatially and electronically. In spite of the numerous advantages of SCCs, these systems have been the least-explored of the supramolecular material categories for biomedical applications, both as drug delivery systems and as anticancer agents.

A specific and attractive area of SCCs is the self-assembly of M_2L_4 (M =metal, L = ligand) metallacages,[9] which can enclose a wide range of small molecules within their cavity, such as ions [10-14] and neutral molecules. [15-21] In addition, the properties of the M_2L_4 coordination cages can be optimized by functionalization of the ligand framework with the aim to target molecular system to a specific cell/tissue type or to enhance detection. Recently, we investigated fluorescent Pd_2L_4 cages (with L being ***exo***-functionalized bipyridyl ligands) as drug delivery systems for cisplatin, which proved to be active in cancer cells, while showing low ***ex vivo*** toxicity in healthy rat liver tissue.[15] The obtained Pd(II) metallacages showed fluorescence properties due to the used ligand system. Similarly, ***exo-***functionalized cages with naphthalene or anthracene groups, or featuring Ru (II) pyridine complexes, were studied with the aim to image their fate in cells via fluorescence microscopy.[22, 23]

Selective accumulation of metallacages in tumors has been hypothesized to occur via the enhanced permeability and retention (EPR) effect,[24] which has been widely used in cancer therapy for delivery via passive targeting. In fact, the EPR effect has been predominantly shown to be involved in the passive targeting of drugs with a molecular weight of more than 40 kDa and for low molecular weight drugs presented in drug-carriers such as polymeric conjugates, liposomes, polymeric nanoparticles, and micellar systems to solid tumors.[25] However, for supramolecular metallacages, with molecular weight of ca. 2-3 KDa, the EPR effect is not likely to influence their delivery. Therefore, it can be assumed that successful conjugation of cell-specific ligands to the cage, including tumor-targeting peptides (TTPs) that are specific for tumor related surface markers, such as membrane receptors,[4, 26]could improve target specificity and efficacy. However, so far this concept has never been explored, and only Fujita *et al.* have been published on the non-covalent peptide coating on self-assembled $M_{12}L_{24}$ coordination spheres.[27]

The synthesis of three Pd_2L_4 cages and their bioconjugation to a model peptide is reported in this work. To the best of our knowledge this is the first attempt to bioconjugate M_2L_4 cages to peptides. The selected cages feature COOH or NH_2 groups in *exo* position for coupling to the peptide by amide bond formation (Fig. 1, **C1a**, **C1b**, **C1c**). It is also investigated whether a longer aliphatic linker between the COOH group and the cage favours coupling of the targeting moiety by reducing possible steric hindrance.

It is worth mentioning that we have opted for this classical bioconjugation method instead of the modern click-chemistry approach, since the latter may lead to interference of Cu^{2+} ions with the stability of the self-assembled cage. In fact, click

chemistry makes often use of copper in the concentration range 50-250 µM or higher,[28] which would be ca. equivalent to the necessary concentration of Pd^{2+} precursor and resulting metallacage, therefore, leading to possible ligand exchange reactions.

The bioconjugation was performed using two different approaches: i) direct tethering of the metallacage to the peptide (**Approach I**); or ii) initial anchoring of the ligand to the peptide, followed by metallacage self-assembly (**Approach II**) (Fig. 1). Formation of the metallacage-peptide constructs was assessed via high-resolution electrospray mass spectrometry in most cases coupled to high performance liquid chromatography (LC-MS). The obtained results are discussed in relation to the advantages and disadvantages of the reported bioconjugation approaches, and constitute the proof of concept for further studies using peptides selected for targeting properties (e.g. cyclic RGD peptides or affimers).

Synthesis

The rigid bidentatepyridyl ligands **L1a**-**L3a** (see Fig. 1) were synthesized using Sonogashira cross-coupling. Reaction of the ligands **L1a**-**L3a** with the Pd precursor $[Pd(NCCH_3)_4](BF_4)_2$ in a 2:1 ratio in DMSO resulted in the coordination cages $[Pd_2(\mathbf{L})_4](BF_4)_4$ **C1a**-**C3a**, respectively, within one hour. The synthesis of the carboxy-functionalized ligand **L1a** and cage **C1a**,[22] as well as of the amine-based ligand **L3a** and cage **C3a** were previously reported,[15] while ligand **L2a** and cage **C2a** were synthesized for the first time following a similar procedure and characterised by NMR and mass spectrometry (Fig. S1-S5 in the Supplementary material). In the ^1H NMR spectrum of **C2a**, the pyridyl proton signals (H_a-H_d) are significantly shifted downfield upon cage formation (Fig. S3). Additional proof for successful cage formation of **C2a** is given by diffusion-ordered NMR spectroscopy (DOSY) revealing a D_{ligand}/ $D_{complex}$ ratio of about 2:1 being in line with literature values.[15] High-resolution ESI–MS analysis of **C2a** shows the expected isotope abundance distribution, with the most intense peaks at *m/z* = 405.8232, 569.7655 and 897.1510, which can be assigned to $[Pd_2(L2a)_4]^{4+}$, $[Pd_2(L2a)_4(BF_4^-)]^{3+}$ and $[Pd_2(L2a)_4(BF_4^-)_2]^{3+}$, respectively (See Fig. S5 and Table S1 in the Supplementary material).

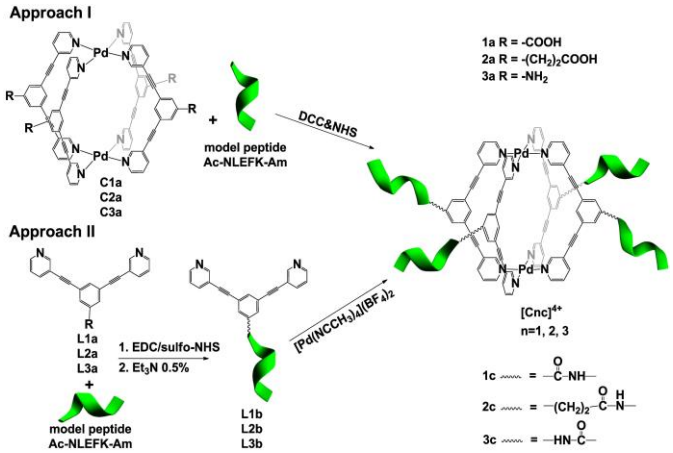

Figure 1. Scheme of the two different bioconjugation approaches applied in this study: i) direct tethering of the metallacage to the peptide (Approach I); or ii) initial anchoring of the ligand to the peptide, followed by metallacage self-assembly *in situ* (Approach II). Theoretically, both approaches can produce bioconjugated Pd_2L_4 cages tethered to four peptide units.

Bioconjugation

Initially, the direct bioconjugation of cages **C1a** and **C2a** to the protected model peptide Ac-NLEFK-Am (acetylated (Ac) at the N-terminal and amidated (Am) at the C-terminus) (**Approach I**, Fig. 1) was attempted via activation of **C1a** or **C2a** with DCC (N',N'-dicyclohexyl carbodiimide) and NHS (N-hydroxysuccinimide) as described in the experimental section. Subsequently, the peptide was added to the intermediate product solution in bicarbonate buffer (pH=9.2) and stirred for 1 h. In the case of the NH_2 *exo*-functionalized cage **C3a**, bioconjugation was carried out by adding EDC to the mixture of model peptide and **C3a** in MES buffer (pH=4.7).

Representative results for cage **C1a** are reported in Fig. S6 in the Supplementary material. The result from MS analysis shows that **Approach I** gives low yield of the bioconjugate product **C1c** (quadruply charged ion *m/z* = 1050.5178). Moreover, a variable number of peptide units were coupled to the cage. Specifically, cages tethered to either one, two or three peptide moieties were detected, corresponding to the most abundant peaks **C1c-1** (triply charged, *m/z* = 727.66), **C1c-2** (quadruply charged, *m/z* = 713.89; triply charged, *m/z* = 951.40)**, C1c-3** (quadruply charged, *m/z* = 882.17; triply charged, *m/z* = 1175.16), respectively. In the MS spectrum, the most abundant peaks were attributed to [**C1c-3+DCC**] species (quadruply charged, *m/z* = 934.02; triply charged, *m/z* = 1244.84), corresponding to one DCC moiety coupled to the carboxylic acid group of the model peptide after formation of **C1c-3**.

Similar results were obtained when bioconjugating cage **C2c**, featuring the longer linker between the cage and the COOH group (data not shown). In the case of cage **C3a**, the activating agent EDC was utilized to promote coupling to the model peptide but most of the peptide appeared to undergo cyclization reactions under these conditions preventing successful bioconjugation.

In general, the obtained results show that it is difficult to both control the number of peptides coupled to the Pd_2L_4 cage and efficiently separate the mixture of different types of bioconjugated cages using **Approach I**.

Therefore, **Approach II** (Fig. 1) was attempted where the carboxylic acid groups of ligands **L1a** or **L2a** were first activated via EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide) and sulfo-NHS (*N*-hydroxysulfosuccinimide) treatment. Afterwards, the coupling reaction was accomplished by incubating the protected model peptide with 0.5% TEA for 0.5 h (pH=7). In the case of the NH_2 *exo*-functionalized ligand **L3a**, bioconjugation was achieved by adding EDC directly to a solution of **L3a** and the model peptide in MES buffer (*pH* = 4.7). The chromatogram obtained to analyze the bioconjugation reaction of ligands **L1a** and **L2a** are depicted in Fig. S7 (panels A and C), and show almost complete conversion of the ligands into the desired products. In fact, **L1b** (**L1a**-peptide) and **L2b** (**L2a**-peptide) are obtained, with a yield higher than 90%. The results show no significant difference in yield of coupling reaction using the ligand with longer aliphatic linker. Fig. S7 (panels B and D) show the MS spectrum of the

bioconjugate products **L1b** (singly charged, *m/z* = 997.45; doubly charged, *m/z* = 499.22) and **L2b** (singly charged, *m/z* = 1025.59; doubly charged *m/z* = 513.32) obtained by ion trap MS.

The amino-functionalized ligand **L3a** forms bioconjugate **L3b** (**L3a**-peptide, singly charged, *m/z* = 968.59; doubly charged, *m/z* = 484.80) less efficiently (singly charged, *m/z* = 968.59; doubly charged, *m/z* = 484.80) most likely due to formation of internal cyclization and dimerization from the model peptide (Fig. S7, panels E and F). Thus, only **L1b** and **L2b** were selected to achieve self-assembly of the bioconjugated cages.

Subsequently, the bioconjugated cages **C1c** or **C2c** were formed *in situ* using a 2:1 ratio of **L1b** or **L2b** and the Pd^{2+} precursor $[Pd(NCCH_3)_4](BF_4)_2$ in DMSO. Representative extracted ion chromatograms and mass spectrum for the bioconjugate cage **C2c** is reported in Figure 2. Fig. S8 in the Supplementary material shows the mass spectrum of bioconjugated cage **C1c**. The results show that both the bioconjugate ligands **L1b** and **L2b** are converted into cage molecules tethered to four peptide units with a yield higher than 95%.

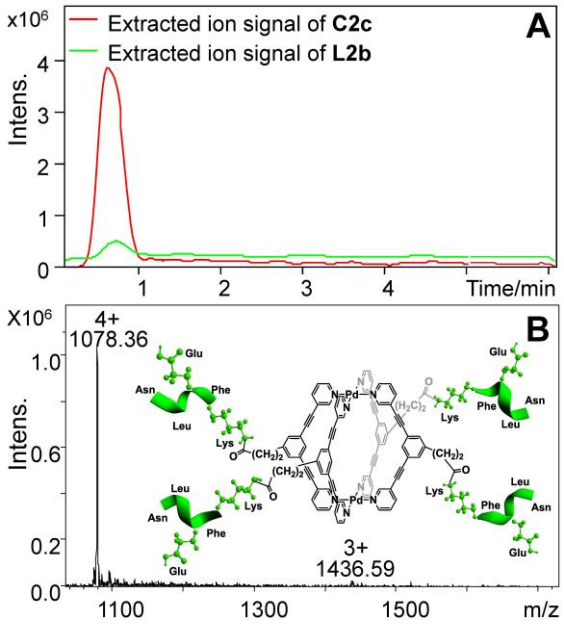

Figure 2. *In situ* self-assembly of the bioconjugated cage **C2c** with peptide Ac-NLEFK-Am analysed by MS with 75% acetonitrile and 0.1% formic acid infused at 50 µL/min. (A) Extracted ion signals of bioconjugated ligand **L2b** (green) and of bioconjugated cage **C2c** (red) at 10 min after self-assembly. (B) Mass spectrum and molecular structure of the bioconjugated product **C2c**.

Table 1. Main peaks identified in the mass spectra of **C2c** and their corresponding CID fragments. The *m/z* values refer to the most intense isotopomer, since the monoisotopic peak has low intensity. No additional peaks were observed in the mass spectrum of the self-assembly reaction of **C2c**.

Reaction and observed ions	*m/z*		Error /ppm	CID fragment
	Measured	Theoretical		
$[C2c]^{4+}$:				**1025.52 (L2b)**
$[Pd_2(L2b)_4]^{4+}$	1078.1852	1078.1839	1.2	1129.48
				1640.11
$[C2c\text{-}H]^{3+}$:				**1025.57 (L2b)**
$[Pd_2(L2b)_4\text{-}H]^{3+}$	1437.2448	1437.2430	1.3	1129.25
				1641.56

The identity of peaks from **C1c** and **C2c** were confirmed by comparison of the experimental and theoretical isotopic patterns, and by CID MS/MS analysis using high resolution MS (Table 1 and Fig. S9 for cage **C2c**, Table S2 and Fig. S10 for cage **C1c**, respectively). Fig. S9 shows that collision induced dissociation (CID) fragmentation of the quadruply charged precursor ion (*m/z* = 1078.19) and triply charged precursor ion (*m/z* = 1437.25) of bioconjugated cage **C2c** leads to dissociation into singly-charged product ions of [**L2b**+H]$^+$ (*m/z* = 1025.52 and *m/z* = 1025.57, respectively). Similarly, Fig. S10 shows that CID fragmentation of the quadruply charged precursor ion (*m/z* = 1050.40) and the triply charged precursor ion (*m/z* = 1399.54) of bioconjugated cage **C1c**, which leads to dissociation into singly charged product ions of [**L1b**+H]$^+$ (*m/z* = 997.45 and *m/z* = 997.24, respectively).

Conclusions

With the aim of implementing supramolecular metallacages as drug delivery systems, we report the first example of bioconjugation of self-assembled Pd_2L_4 cages to the model linear peptide Ac-NLEFK-Am. The obtained results open the possibility of efficient bioconjugation of metallacages to peptides which could be extended to targeting moieties such as cyclic RGD peptides or affimers, and possibly also to antibodies. This opportunity is particularly attractive in the case of metallacages encapsulating anticancer drugs (e.g.: cisplatin) in order to efficiently target them to cancer cells. Two approaches of bioconjugation of metallocages to peptides have been attempted, both based on amide bond formation between the carboxylic acid (or amine) serving as *exo*- functionalized ligand/cage and the amine (or carboxylic acid) groups of the model peptide side chains. So far the best results were achieved with **Approach II**, where first the coupling of the peptide to the ligands constituting the cages was performed, followed by ***in-situ*** reconstitution of the Pd_2L_4 cages via self-assembly. No major advantages were noticed in the use of a long-linker COOH moiety for bioconjugation in both approaches. Instead, improved bioconjugation efficiency was observed in the case of the ***exo***-functionalization with carboxylic acids compared to amino groups. In the latter case, formation of peptide cyclic by-products prevented efficient bioconjugation under the applied reaction conditions. Nevertheless, NH_2 functionalization may still be suitable for bioconjungation of the cages with peptides of different sequences and with antibodies, and will certainly be considered in future studies.

Future research in our group will focus on tethering Pd_2L_4 cages to targeting peptides and to investigate the activity of the supramolecular bioconjugates in cancer cells and tissues.

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Notes and references

1 J. K. Vasir and V. Labhasetwar, *Technology in Cancer Research & Treatment*, 2005, **4**, 363-374.

2 A. Z. Wang, R. Langer and O. C. Farokhzad, *Annual Review of Medicine, Vol 63*, 2012, **63**, 185-198.

3 F. Meng, N. Han and Y. Yeo, *Expert Opinion on Drug Delivery*, 2016, DOI: 10.1080/17425247.2016.1218464, 1-20.

4 X. Y. Wang, X. H. Wang and Z. J. Guo, *Accounts of Chemical Research*, 2015, **48**, 2622-2631.

5 J. S. Butler and P. J. Sadler, *Current Opinion in Chemical Biology,* 2013, **17**, 175-188.

6 M. Nave, R. E. Castro, C. M. Rodrigues, A. Casini, G. Soveral and M. M. Gaspar, *Nanomedicine (Lond)*, 2016, **11**, 1817-1830.

7 M. J. Webber, E. A. Appel, E. W. Meijer and R. Langer, *Nature Materials*, 2016, **15**, 13-26.

8 T. R. Cook, V. Vajpayee, M. H. Lee, P. J. Stang and K. W. Chi, *Accounts of Chemical Research*, 2013, **46**, 2464-2474.

9 A. Schmidt, A. Casini and F. E. Kuhn, *Coordination Chemistry Reviews*, 2014, **275**, 19-36.

10 R. Sekiya, M. Fukuda and R. Kuroda, *Journal of the American Chemical Society*, 2012, **134**, 10987-10997.

11 M. X. Han, R. Michel, B. He, Y. S. Chen, D. Stalke, M. John and G. H. Clever, *Angewandte Chemie-International Edition*, 2013, **52**, 1319-1323.

12 G. H. Clever, W. Kawamura, S. Tashiro, M. Shiro and M. Shionoya, *Angewandte Chemie-International Edition*, 2012, **51**, 2606-2609.

13 S. Freye, J. Hey, A. Torras-Galan, D. Stalke, R. Herbst- Irmer, M. John and G. H. Clever, *Angewandte Chemie-International Edition*, 2012, **51**, 2191-2194.

14 S. Freye, R. Michel, D. Stalke, M. Pawliczek, H. Frauendorf and G. H. Clever, *Journal of the American Chemical Society*, 2013, **135**, 8476-8479.

15 A. Schmidt, V. Molano, M. Hollering, A. Pothig, A. Casini and F. E. Kuhn, *Chemistry-a European Journal*, 2016, **22**, 2253-2256.

16 N. Kishi, M. Akita and M. Yoshizawa, *Angewandte Chemie- International Edition*, 2014, **53**, 3604-3607.

17 N. Kishi, Z. O. Li, K. Yoza, M. Akita and M. Yoshizawa, *Journal of the American Chemical Society*, 2011, **133**, 11438-11441.

18 J. E. M. Lewis, E. L. Gavey, S. A. Cameron and J. D. Crowley, *Chemical Science*, 2012, **3**, 778-784.

19 P. H. Liao, B. W. Langloss, A. M. Johnson, E. R. Knudsen, F. S. Tham, R. R. Julian and R. J. Hooley, *Chemical Communications*, 2010, **46**, 4932-4934.

20 S. Loffler, J. Lubben, L. Krause, D. Stalke, B. Dittrich and G. H. Clever, *Journal of the American Chemical Society*, 2015, **137**, 1060-1063.

21 M. Yamashina, M. M. Sartin, Y. Sei, M. Akita, S. Takeuchi, T. Tahara and M. Yoshizawa, *Journal of the American Chemical Society*, 2015, **137**, 9266-9269.

22 A. Schmidt, M. Hollering, M. Drees, A. Casini and F. E. Kuhn, *Dalton Transactions*, 2016, **45**, 8556-8565.

23 A. Schmidt, M. Hollering, J. Han, A. Casini and F. E. Kuhn, *Dalton Trans*, 2016, **45**, 12297-12300.

24 B. Therrien, *Top Curr Chem*, 2012, **319**, 35-55.

25 H. Maeda, J. Wu, T. Sawa, Y. Matsumura and K. Hori, *Journal of Controlled Release*, 2000, **65**, 271-284.

26 R. Haubner, D. Finsinger and H. Kessler, *Angewandte Chemie International Edition in English*, 1997, **36**, 1374-1389.

27 M. Ikemi, T. Kikuchi, S. Matsumura, K. Shiba, S. Sato and M. Fujita, *Chemical Science*, 2010, **1**, 68-71.

28 S. I. Presolski, V. P. Hong and M. G. Finn, *Curr Protoc Chem Biol*, 2011, **3**, 153