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

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Supramolecular Pd2L4 cages (L = ligand) hold promise as drug delivery systems. With the idea of achieving targeted delivery of the metallo-cages to tumor cells, the bioconjugation of exo-functionalized self-assembled Pd2L4 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. In some cases, it was also possible to achieve a synergistic anticancer effect of different therapeutic modalities combined in one drug delivery system. 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. 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.

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 metallo-cycles 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. 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 M2L4 (M = metal, L = ligand) metallacages, which can enclose a wide range of small molecules within their cavity, such as ions and neutral molecules. In addition, the properties of the M2L4 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 Pd2L4 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. The obtained Pd(n) metallacages showed fluorescence properties due to the used ligand system. Similarly, exo-functionalized cages with naphthalene or anthracene groups, or featuring Ru(n) pyridine complexes, were studied with the aim to image their fate in cells via fluorescence microscopy.

† Electronic supplementary information (ESI) available: Experimental, NMR and MS spectra. See DOI: 10.1039/c6cc08937b
Selective accumulation of metallacages in tumors has been hypothesized to occur via the enhanced permeability and retention (EPR) effect, 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. 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 recognize tumor related surface markers, such as membrane receptors, 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.

The synthesis of three $Pd_4L_4$ cages and their bioconjugation to a model peptide are reported in this work. To the best of our knowledge this is the first attempt to bioconjugate $M_{12}L_{24}$ 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, C1, C2, C3). 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 mM or higher, 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 in situ (Approach II). Theoretically, both approaches can produce bioconjugated $Pd_4L_4$ cages tethered to four peptide units.

![Fig. 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 $Pd_4L_4$ cages tethered to four peptide units.](image-url)
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 Pd4L4 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-hydroxy succinimide) 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 NH2-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 is depicted in Fig. S7 (panels A and C) (ESI†), 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) (ESI†) 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, ESI†). 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 Pd2+ precursor [Pd(NCCCH3)4][BF4]2 in DMSO. Representative extracted ion chromatograms and mass spectrum for the bioconjugate cage C2c is reported in Fig. 2. Fig. S8 in the ESI† 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%.

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, ESI†). Fig. S9 (ESI†) shows that collision induced dissociation (CID) fragmentation of the quadruply charged precursor ion (\( m/z = 1078.19 \)) and triply charged precursor ion (\( m/z = 1437.28 \)) 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 (ESI†) 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).

With the aim of implementing supramolecular metallacages as drug delivery systems, we report the first example of bioconjugation of self-assembled Pd4L4 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 metallacages 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 reconstruction of the Pd$_2$L$_4$ 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 were noticed in the use of a long-linker COOH moiety for bioconjugation under the applied reaction conditions. Nevertheless, NH$_2$ substitution of the Pd$_2$L$_4$ cages to targeting peptides and to investigate the activity of the supramolecular bioconjugates in cancer cells and tissues.

Future research in our group will focus on tethering Pd$_2$L$_4$ cages to targeting peptides and to investigate the activity of the supramolecular bioconjugates in cancer cells and tissues.

A. C. acknowledges support from the Hans Fischer Senior Fellowship of the Technical University of Munich – Institute for Advanced Study, funded by the German Excellence Initiative and the European Union Seventh Framework Programme under grant agreement no. 291763. A. S. is grateful to the Boehringer-Ingelheim Fonds and to the TUM Graduate School of Chemistry for financial support. We gratefully acknowledge the China Scholarship Council (CSC) for a PhD fellowship to J. H. J. H. wishes to thank Jos Hermans, Sara Ongay and Marcel de Vries for helping with the LC-MS system.

Notes and references