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 metallacages 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. 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 metallacycles 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 M$_2$L$_4$ (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 M$_2$L$_4$ 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$_2$L$_4$ 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. 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.

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 to solid tumors. 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, 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$_2$L$_4$ 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$_2$L$_4$ 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, which would be ca. equivalent to the necessary concentration of Pd²⁺ 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(NCCH₃)₄][BF₄]₂ in a 2:1 ratio in DMSO resulted in the coordination cages [Pd₂(La)₄][BF₄]₄ C1a-C3a, respectively, within one hour. The synthesis of the carboxy-functionalized ligand L1a and cage C1a, as well as of the amine-based ligand L3a and cage C3a were previously reported, 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 (Hₐ-Hₐ) 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 Dligand/ Dcomplex ratio of about 2:1 being in line with literature values. 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 [Pd₂(La)₄]⁴⁺, [Pd₂(La)₄(BF₄)⁺]³⁺ and [Pd₂(La)₄(BF₄)₂]²⁺, 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$_2$L$_4$ 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$_2$L$_4$ 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.

<table>
<thead>
<tr>
<th>Reaction and observed ions</th>
<th>m/z Measured</th>
<th>m/z Theoretical</th>
<th>Error /ppm</th>
<th>CID fragment</th>
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<tbody>
<tr>
<td>[C2c]^{4+}</td>
<td></td>
<td></td>
<td></td>
<td>1025.52 (L2b)</td>
</tr>
<tr>
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<td>1641.56</td>
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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 Pd2L4 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 Pd2L4 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, NH2 functionalization may still be suitable for bioconjugation 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$_2$L$_4$ 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**


