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Research paper

Formation of platinum (II) as a six member ring for sustained polymeric delivery

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ABSTRACT

A new pH-activated polymer chelate of cisplatin was synthesized using a scalable and green aqueous technique. Synthesis of the chelate was based on formation of a 6-member ring of platinum(II) with acetyl-homo-Lysine (Ac-homo-Lys), which was accomplished under completely aqueous conditions using a traceless photocleavable protection chemistry. Synthesis preceded by, first, amidation of a photocaged homo-Ac-Lys with hyaluronic acid (HA) in water using a *p*-hydroxyphenacyl (pHP) group as the photoremovable protecting group, followed by reaction of cisplatin (diaqua form) in water to form the reversible chelate. Platinum drug release was pH rate controlled, with more rapid release ($t_{1/2}$ 20 h) at acidic pH similar to the tumor microenvironment yet slower release ($t_{1/2}$ 35 h) at normal physiological pH.

1. Introduction

Platinum therapies are part of most first or second-line chemotherapeutic regimens due to the efficacy of these DNA-crosslinkers in a broad number of malignancies, and over half of cancer patients receive cisplatin or one of its analogues [1]. Cisplatin has substantial renal and peripheral neural toxicity that has been partially addressed with the FDA-approved analogues carboplatin and oxaliplatin; however, neither has improved on cisplatin's efficacy and at best the side-effects are redirected to other organ systems.

Current platinum drugs are not targeted to cancer cells or tissues. Cisplatin and its analogues differ in their rates of hydrolytic activation to form the active DNA-reactive species and their relative cell permeability [2,3], which results in differences in pharmacokinetics and tissue distribution [4,5]. However, any selectivity to cancer cells is largely a result of the more efficient DNA break repair and slower replication of normal cells. The targeting of platinum drugs to cancers may substantially increase the drug penetration into tumor tissue and metastatic lymph nodes, as well as reducing the off-target toxicities of the chemotherapeutic agents that often limit their clinical use. Targeting may be achieved by utilizing a carrier-based drug delivery platform such as hyaluronan (HA) conjugated cisplatin as HA is the primary ligand for the CD44 receptors that are often highly expressed on the surface of human cancer cells.

Controlled release of drugs is also an important strategy, not only to prolong drug action by providing constant doses coupled with tunable release over long periods, but also to permit maintenance of prescribed drug levels within a therapeutic window - thus minimizing deleterious effects of the drug after administration. Conventional platinum therapies are given intravenously, which results in the rapid clearance of the drug from the body and minimum retention in diseased organs. We have a longstanding interest in developing strategies for targeted delivery and the controlled release of cancer drugs including cisplatin [6,7]. Our previous studies have shown that sustained released drug delivered by nanocarriers via local regional injection demonstrated superior pharmacokinetics, reduced side effects and significantly improved anti-cancer efficacy in various mouse xenografts and naturally occurring canine cancers. Herein, we discuss the molecular modeling studies, synthesis (Scheme 1), and reaction kinetics of five-membered ring and six-membered ring formation of cisplatin chelates with HA-Lys in water. A key feature of our approach is generating the cisplatin chelates in water using a photoremovable protecting group (PPG) [8,9]. These analogues substantially sustain the release kinetics of cisplatin from the carrier and result in more sustained delivery of active drugs.

Abbreviations: HA, hyaluronic acid; PPG, photoremovable protecting group; pHP, *p*-hydroxyphenacyl; Fmoc, fluorenylmethoxy-carbonyl; HATU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate; DMTMM, 4-(4,6-Dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride; DIEA, *N*,*N*-diisopropylethylamine; TFA, trifluoroacetic acid; DCM, Dichloromethane; PBS, Phosphate-buffered saline

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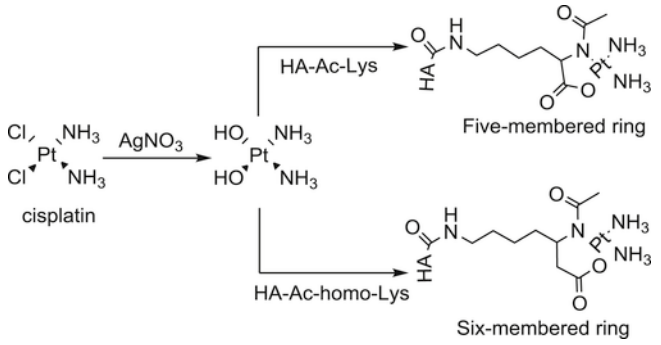


Scheme 1. Synthesis of HA-Lys-Pt via five-membered and six-membered rings.

2. Results and discussion

2.1. Molecular modeling studies

Molecular modeling studies were initially performed to probe the feasibility of five-membered and six-membered ring formation with cisplatin using Spartan software (version 14) in vacuum (Fig. 1). In silico calculations for the energy minimized structures showed that heat of formation for the ring generation with cisplatin is almost identical (five-membered ring; −703 hartrees vs −742 hartrees for the six-membered ring). Therefore, we performed the synthesis of these second-generation ring-conjugates in plain water to identify the more readily formed and sustained released cisplatin conjugate for in vitro and in vivo studies.

2.2. Chemistry

The HA-Ac-Lys-Pt conjugate (with a five-membered ring) and HA-Ac-homo-Lys-Pt conjugate (with a six-membered ring) were prepared as illustrated in Schemes 2 and 3. Synthesis of the HA-Ac-Lys-Pt conjugate **7** was accomplished by first amidation of the caged Ac-Lys with HA in water using a *p*-hydroxyphenacyl (pHP) group as the photoremovable protecting group, followed by reaction of cisplatin (diaqua form) in water. The synthesis was begun with readily available starting material, methyl 5-acetylsalicylate **1**. α-Bromination of **1** with copper (II) bromide gave **2** (85%), which was then reacted with Ac-Lys(Boc)-OH in MeCN to afford pHP caged Ac-Lys(Boc), **3**. Boc deprotection gave pHP-Ac-Lys **4** in good yield (85%) which was reacted with HA (75 kDa) in water to afford HA-pHP-Ac-Lys conjugate **5**. The pHP group was removed by photolysis in water at ~300–350 nm. The resulting photoproducts were removed by dialysis to afford HA-Ac-Lys **6**, which was then converted to the HA-Ac-Lys-Pt conjugate **7** at 50 °C, using cisplatin (diaqua form) as reported previously [6].

Synthesis of HA-Ac-homo-Lys-Pt conjugate **15** was performed using a similar approach as described above. Briefly, pHP caged Fmoc-homo-Lys(Boc)-OH **8** was synthesized from the pHP α-bromo analog **2** in good yields (86%). Fmoc deprotection with care gave **9**, which was reacted with acetyl chloride to generate the acetyl protected analog **10** (92%). After removing the Boc protection, pHP-Ac-homo-Lys [mixture of **11** (~80%) and **12** (minor)] was reacted with HA to afford HA-pHP-Ac-homo-Lys, **13**. Photolysis followed by dialysis was performed to generate HA-Ac-homo-Lys, **14**. Resulting conjugate, **14** was then reacted with cisplatin (diaqua) to produce the final product of HA-Ac-homo-Lys-Pt conjugate, **15** as described above.

In both cases, a change in color from yellow to beige and fall in pH during the coupling of Pt-diaqua to HA-Lys were useful to monitor the kinetics of the drug conjugate formation (**7** vs. **15**). Noticeable beige coloration was observed in the reaction mixture for the synthesis of drug conjugate 15 overnight, whereas it took more than 24 h for drug conjugate **7** to develop a similar appearance in the reaction mixture. A sharp initial drop in pH occurred for both reactions, which was adjusted back to ca. pH 5 with NaOH. The reaction mixture of conjugate 15 required several more additions of NaOH over 48 h to maintain a pH of ca. 5, whereas the reaction pH of conjugate 7 was nearly constant for 36 h. The Pt loading degrees of conjugates 7 and 15 were determined to be 5% and 6%, respectively, by inductively coupled plasma mass spectrometry (Agilent 7500a ICP-MS, method described in Ref. [6]).

In our design of new sustained release of drug conjugates using a ringer linker chemistry, we attempted to avoid or circumvent the inherent instability of direct conjugation of cisplatin onto the carboxylates of hyaluronic acid, as the resulting conjugate had a short release half-life (10 h in PBS) in our previous studies [10]. Our ultimate goal as to develop a rapid and scalable synthetic protocol to generate HA-Lys-Pt conjugates in water for drug delivery applications, in which the HA-Lys-Pt conjugates demonstrate favorable pharmacokinetic properties and slower release rates in vivo. As far as synthesis of these HA-based drug conjugates are concerned, it was a prudent choice to select linker chemistry, which involves not only milder and faster reaction conditions, but also potential for scale-up to the clini-

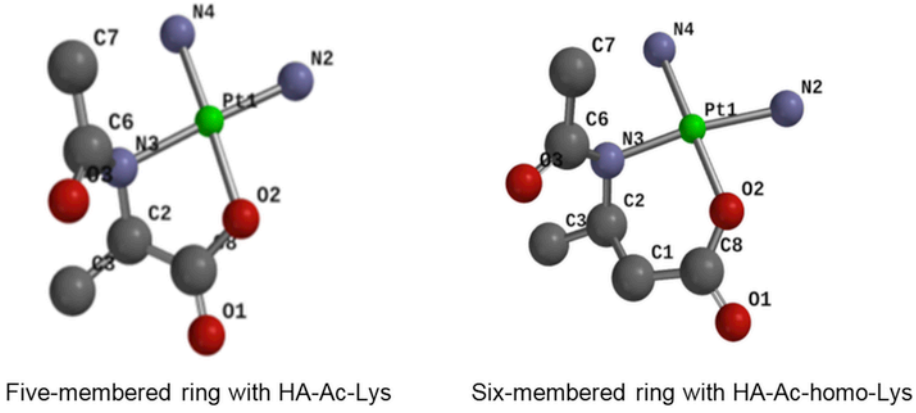
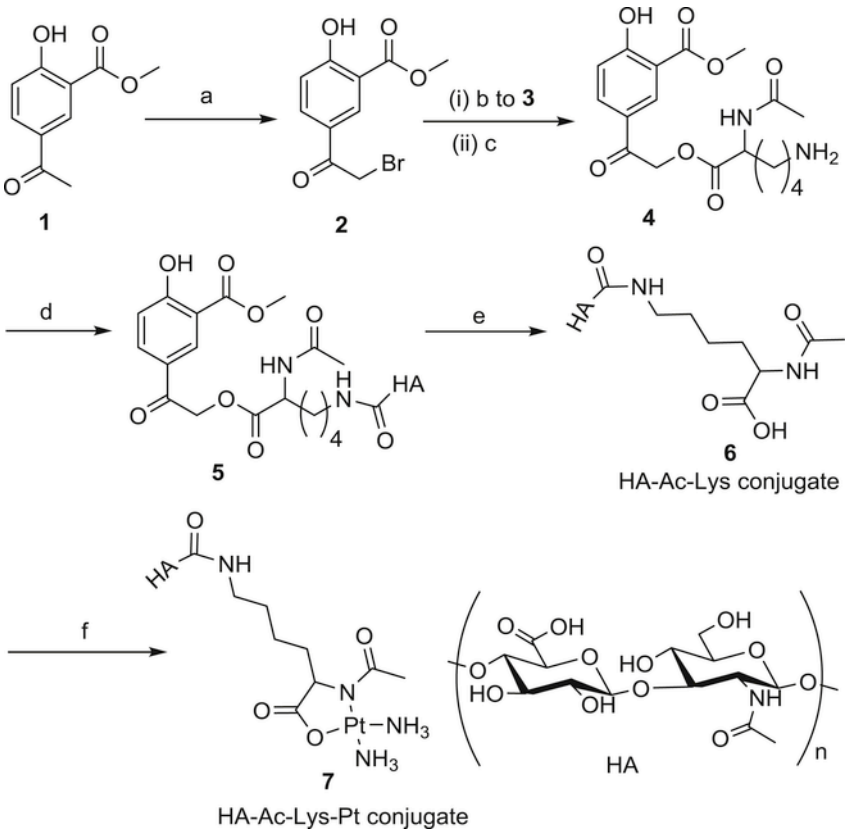


Fig. 1. Energy minimized structures of five-membered ring versus six-membered ring with cisplatin (for clarity, alkyl side chains of lysine and homo-lysine have been removed and represented as C3).



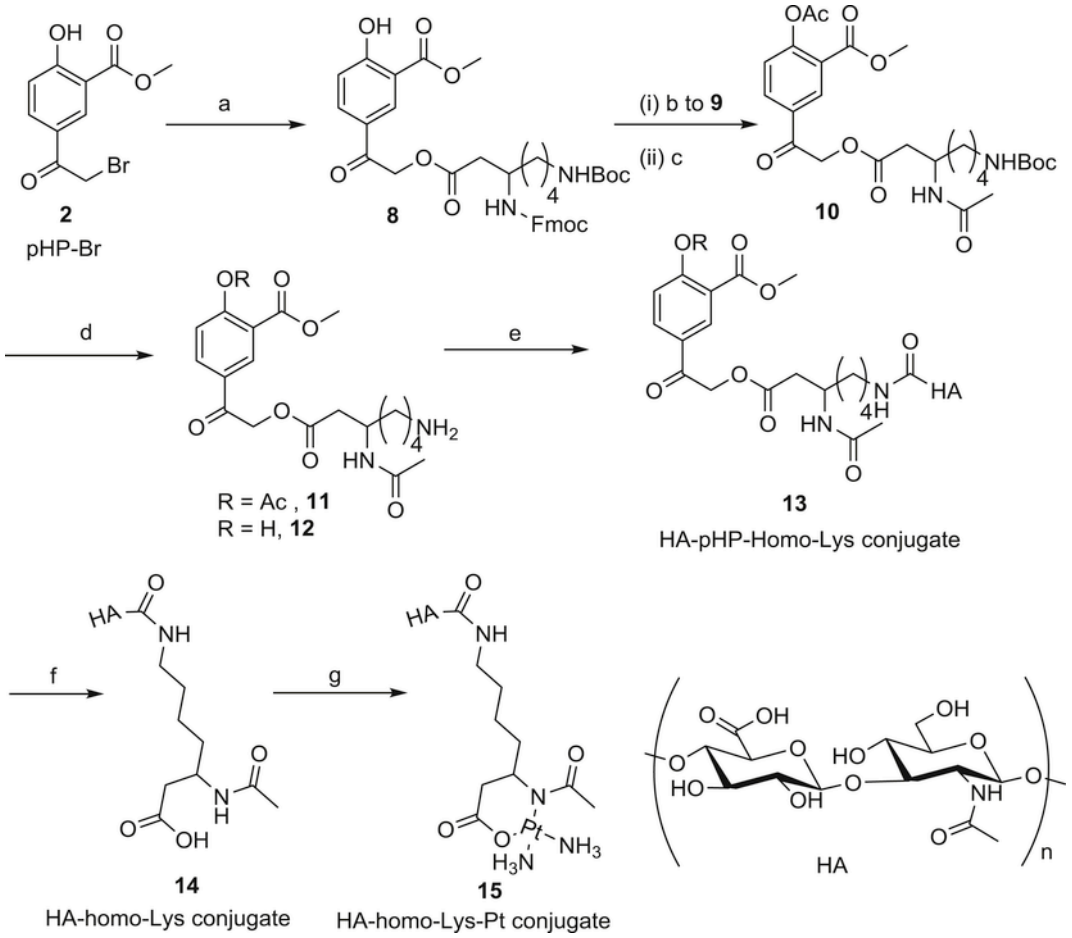
Scheme 2. Synthesis HA-Ac-Lys-Pt conjugate; Reagents and conditions: a) $CuBr_2$, $CHCl_3$/EtOAc, 40–50 °C, 3–4 h, 85%; b) Ac-Lys(Boc)-OH, K_2CO_3, MeCN, overnight; c) TFA/DCM, 2–3 h, 85%; d) HA, DMTMM, H_2O, 37 °C, 48 h, pH ~4–5, 20 mg/mL; e) hv, photolysis, H_2O, 2–4 h; f) $Pt[H_2O]_2[NH_3]_2$, H_2O, pH ~5, 50 °C, 48 h.

cal scale. Since the hydrophilic and biodegradable polymer HA is pH sensitive, acid and base labile protecting groups could not be utilized effectively. In this study, we successfully demonstrated that the use of photoremovable protecting groups (PPG; e.g., *p*-hydroxyphenacyl group, pHP) in the synthesis of drug conjugates **7** and **15** [8,9]. In this process, light is considered as a "traceless" reagent to release the substrate [11] (e.g., HA-Lys). pHP is an emerging PPG with advantageous qualities for these syntheses, mainly due to its properties of: 1) absorption at wavelengths near or above 400 nm; 2) enhanced chemical and photochemical quantum yields; 3) improved rate of release, ideally in the range of picoseconds to nanoseconds time constants; 4) good water solubility; and 5) its clean photochemistry. In this study we choose a derivative of pHP, methyl 5-acetylsalicylate, to induce intramolecular H-bonding between hydroxyl group and carbonyl of the ester functionality (Fig. 2). Therefore, this H-bonding interaction reduced the unwanted coupling of phenolic hydroxyl group with the carboxylic acid functional group of HA during the amidation reaction.

Previously we reported that HA-Ac-Lys could be utilized to conjugate cisplatin forming a robust five-membered ring with the COOH group and nitrogen of N-acetyl group of lysine linker [6]. However, this method involved the reaction between HA-tetrabutylammonium salt and N-Ac-Lys in DMSO, which is difficult to scale up and time-consuming to purify. Industrial scale batches of polymer drug conjugates are typically produced under aqueous condition and purified and concentrated using a tangential flow filtration system. Therefore, we have developed the syntheses discussed in this paper in water as an improved method, which involves a PPG in the synthesis. To further improve the reaction times and Pt loading degree, we have attempted to optimize the synthesis using Ac-homo-Lys as the linker, which forms a six-membered ring with cisplatin. Six-membered non-aromatic heterocyclic compounds are generally more stable than that of five-membered counterparts, as the six-membered non-aromatic rings are in chair conformation in which there is minimal angle and eclipse strain. Bond angles for six-membered ring are closer to the tetrahedral angle, which eliminates the ring strain imparting more stability. It is reported that ring strain of platinacyclohexane is smaller than that of five-membered platinacycloalkanes based on studies of cyclometalation of dialkylbis(triethylphosphine)platinum(II) complexes [12]. Moreover, Schwartz and co-workers showed that six-membered chelate ring formed by salicylate and platinum exhibited greater stability. The six-membered chelate ring of salicylate and platinum was stable for days in solution [13].

Release studies of platinum species from HA-Lys-Pt [6] and HA-homo-Lys-Pt were evaluated via dialysis against PBS at the physiological pH of 7.4 at 37 °C and against acetate buffer at pH 5.5 to mimic the acidic environment of tumor cells, which is normally 6.5–6.8 (normal physiological pH is 7.3–7.4) but may be < pH 5 in necrotic regions [14]. The platinum concentrations in dialysis bag were measured and plotted as the percentage of cumulative drug released against time (Fig. 3). The drug release from HA-Lys-Pt exhibited a half-life of 24 h (data from Ref. [6]) whereas HA-homo-Lys-Pt exhibited a longer half-life of ~35 h. These data indicate that the six-membered ring with Pt-diaqua is more stable than that of the five-membered ring.

Drug release was more rapid in acetate buffer with a half-life of 32 h for HA-Lys-Pt [6], whereas the half-life is 20 h for HA-homo-Lys-Pt (Fig. 3). The rapid release of platinum ions was due to the faster protonation of N^{α}-acetylamido ligand under acidic conditions, followed by de-chelation from the Pt(II). The faster release of Pt-di-



Scheme 3. Synthesis HA-Ac-homo-Lys-Pt conjugate; Reagents and conditions: a) Fmoc-homo-Lys(Boc)-OH, K_2CO_3, MeCN, overnight, 86%; b) Piperidine, DMF, 0 °C, 30 min; c) CH_3COCl, CH_2CL_2, Et_3N, 0 °C, 30 min, 92%; d) TFA/DCM, 2–3 h, **11**–80%; e) HA, DMTMM, H_2O, 37 °C, 48 h, pH ∼4–5, 20 mg/mL; f) hv, photolysis, H_2O, 2–4 h, 100%; g) $Pt[OH]_2[NH_3]_2$, H_2O, pH ∼5, 50 °C, 48 h.

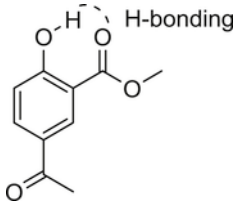


Fig. 2. Intramolecular H-bonding of methyl 5-acetylsalicylate.

aqua complex at lower pH may be advantageous to the formation of Pt-DNA adducts in tumor cells [6]. Therefore, drug release through a six-membered ring is an added advantage with relatively little burst release of drug during the entire study period.

The anti-proliferative activity of HA-homo-Lys-Pt was evaluated using human head and neck squamous cell carcinoma (HNSCC) cell line, MDA-1986 and two human breast cancer lines (Supplementary Fig. 19). Cisplatin and the 5-member ring analog HA-Lys-Pt were used as controls. Both conjugates and cisplatin show full anti-proliferative efficacy in MDA-1986 cells. The HA-Homo-Lys-Pt conjugate is slightly less potent (IC_{50} ≅ 12 μM) than cisplatin (IC_{50} ≅ 10 μM). The reduced IC_{50} of the conjugates compared to cisplatin is attributed to the extended release of the active forms of Pt species from the conjugate, so that during the 72 h cell toxicity study, only a limited portion of the Pt from conjugates was available. The HA-Homo-Lys-Pt

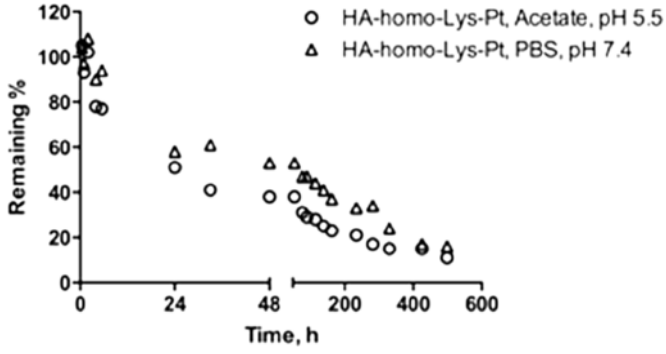


Fig. 3. *In vitro* release of Platinum species from HA-homo-Lys-Pt in PBS or acetate buffer and 37 °C.

exhibits higher growth inhibition potency than the HA-Lys-Pt conjugate (IC_{50} ≅ 40 μM). This is due to the faster release of the free drug from the HA-Homo-Lys-Pt conjugate in the acidic environment of cell endosomes ($t_{1/2}$ = 20 h at pH 5.5), compared to HA-Lys-Pt ($t_{1/2}$ = 32 h at pH 5.5). In breast cancer cell lines MDA-MB-231 and MCF7, the HA-Homo-Lys-Pt had an IC_{50} of 70 μM and 44 μM, respectively, compared to cisplatin's IC_{50}'s of 9.1 μM and 12 μM in these cell lines.

3. Conclusions

In summary, the drug conjugates **7** and **15** were successfully synthesized in water with high Pt loading degrees. The Pt-diaqua drug conjugate containing a six-membered ring **15** was successfully synthesized for the first time. The formation of six-membered ring (**15**) with Pt-diaqua is faster than that of five-membered ring (**7**) formation. This optimized synthetic protocol has the potential to generate cisplatin drug conjugates in kilogram scales to provide high drug loading degrees to meet the demand for these HA-Lys-Pt conjugates for various chemotherapeutics applications. Further studies on these drug conjugates are currently underway in our laboratory.

4. Experimental

Detailed synthesis procedures for compounds **2**–**15**, NMR and MS characterization of these compounds can be found in the Supporting Information.

Notes

TZ and CG are employees of HylaPharm; LF has ownership interest in HylaPharm, which has licensed portions of this technology from KU. SNS declares no competing financial interest.

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Appendix A. Supplementary data

Supplementary data related to this chapter can be found at http://dx.doi.org/10.1016/j.ejmech.2017.05.020.

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