Pt-induced crosslinks promote target enrichment and protection from serum nucleases

Rachael M. Cunningham[a,b], *Anna M. Hickey*[a], *Jesse W. Wilson*[a,b], *Kory J. I. Plakos*[a,b], *and Victoria J. DeRose*[a,b*]

[a]Department of Chemistry and Biochemistry, University of Oregon, Eugene, OR 97403, USA

[b]Institute of Molecular Biology, University of Oregon, Eugene, OR 97403, USA

*Corresponding author. V. DeRose, email: derose@uoregon.edu, phone:

Abstract

Identifying the interactions of small molecules with biomolecules in complex cellular environments is a significant challenge. As one important example, despite being widely used for decades, much is still not understood regarding the cellular targets of Pt(II)-based anticancer drugs. In this study we introduce a novel method for isolation of Pt(II)-bound biomolecules using a DNA hybridization pull-down approach. Using a modified Pt reagent, click-ligation of a DNA oligonucleotide to both a Pt(II)-bound DNA hairpin and bovine serum albumin (BSA) are demonstrated. Subsequent hybridization to a biotin-labeled oligonucleotide allows for efficient isolation of Pt(II)-bound species by streptavidin pulldown. We also find that platinated bovine serum albumin readily crosslinks to DNA in the absence of click ligation, and that a fraction of BSA-bound Pt(II) can transfer to DNA over time. Interestingly, in in vitro studies, fragmented mammalian DNA that is crosslinked to BSA through Pt(II) exhibits significantly increased protection from degradation by serum nucleases.

1. Introduction

Identifying the interactions of small molecules such as metal-ligand complexes in complex cellular environments is a significant challenge. Because of this, drug discovery often relies on phenotypic screening for identification of novel small molecules without knowledge of all cellular interactions. While many potent bioactive compounds have been developed using this approach, it lacks valuable information about the cellular targets underpinning efficacy in addition to those contributing to off-target effects [1,2]. Thus, for many small molecule drugs that are commonly used today, a full understanding of their mechanism of action is not available [3]. Through understanding small molecule target

interactions, more precise cellular mechanisms can be elucidated and compounds with increased specific activity and fewer side effects can be developed.

Pt(II)-based anticancer therapeutics such as cisplatin [4] are one such group of small molecules. Despite their prevalent use, the clinical efficacies of cisplatin and its two FDA-approved derivatives—carboplatin and oxaliplatin—are hindered by acquired resistance and many side effects [5]. It is generally accepted that one major mechanism of action for these Pt(II)-based compounds is through interactions with DNA, which lead to inhibition of replication and ultimately cell death [6]. Additionally, interest in non-DNA targets of Pt(II)-based drugs has increased in recent years with evidence of significant Pt(II) interactions with cellular RNA [9-13] and proteins [14,15]. As cisplatin has been shown to initiate apoptosis independent of nuclear DNA [16,17], these alternative targets of Pt(II) are hypothesized to play important roles in Pt(II)-induced cell death.

Ultimately, comprehensive identification of Pt-bound biomolecules obtained from treated cells is desired. Various methods have been proposed to specifically isolate and enrich for Pt(II)-bound molecules from unmodified components in the cell [18,19]. Two recent reports identified Pt(II)-DNA adducts genome-wide through pulldown of high mobility group protein HMGB1, which is well known to bind Pt(II)-DNA adducts [7], or using antibodies that recognize [$(NH_3)_2$Pt]-DNA adducts [8]. These methods have provided important identification about binding partners for a selection of Pt-DNA species. Another recent study used high-throughput sequencing to predict major Pt-RNA adduct sites on ribosomal RNA based on the ability of Pt-RNA adducts to inhibit reverse transcription [13]. While powerful, this method is an indirect readout of possible Pt adducts and is challenged by the sparse number of ‘hits’ in a vast sequence space. Methods to enrich Pt-bound species before analysis would greatly benefit this and similar approaches.

Various methods have been proposed to specifically isolate and enrich for Pt(II)-bound molecules from unmodified components in the cell [18,19]. One comprehensive method of selecting and enriching Pt-bound species from a complex mixture would be to use a Pt(II) reagent that can be easily labeled once already bound to its target. To this end, we have synthesized a suite of azide- and alkyne-modified Pt(II) compounds that can participate in the Cu(I) catalyzed alkyne-azide cycloaddition "click" reaction (Figure 1). These compounds allow for labeling and enrichment of targeted cellular biomolecules post-treatment.

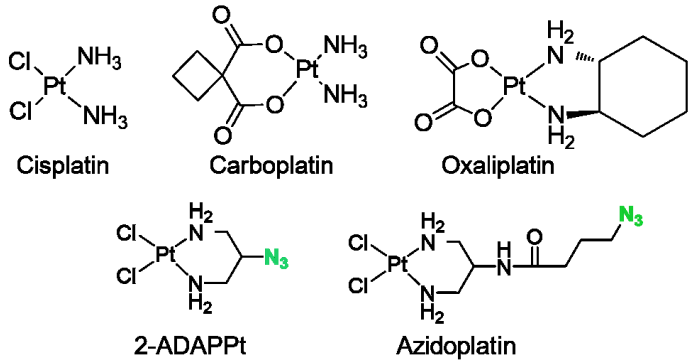

Figure 1. FDA-approved Pt(II) based drugs (top) and the azide-modified Pt(II) compounds used in this study (bottom).

We have previously demonstrated the utility of our click-enabled Pt(II) compounds *in vitro* and *in cellulo* by directly labeling Pt(II)-bound molecules with a fluorophore or biotin [15,20-22]. Here, we expand this toolbox using a modified DNA hybridization approach [23] to detect Pt(II) adducts formed on biomolecules following treatment with our azide-modified Pt(II) compounds. In this approach, instead of directly labeling the Pt(II)-bound molecule with biotin, a hexynyl-modified DNA oligonucleotide (hex-DNA) is first click-conjugated. The attached hex-DNA is then hybridized to a biotin-modified DNA oligonucleotide (bio-DNA) and the resulting complex is incubated with streptavidin beads to select Pt-bound species. The Pt(II)-bound molecules can be released from the bead by heating above the melting point of the hybridized DNA oligomers (Figure 2).

It has been shown that increasing linker length between the biotin tag and molecule of interest greatly increases the binding capacity of streptavidin [24]. Although the standard biotin alkyne used for click-conjugation contains a polyethylene glycol linker, conjugating the targeted biomolecule to a DNA oligonucleotide further distances the biotin tag from the Pt(II)-bound biomolecule (Figure 2). We predict that increasing the linker length between the Pt(II) adduct and biotin using our DNA labeling method will also increase pull-down yield over direct biotinylation. Additionally, this method covalently attaches a versatile DNA tag that can be used in downstream applications following affinity purification.

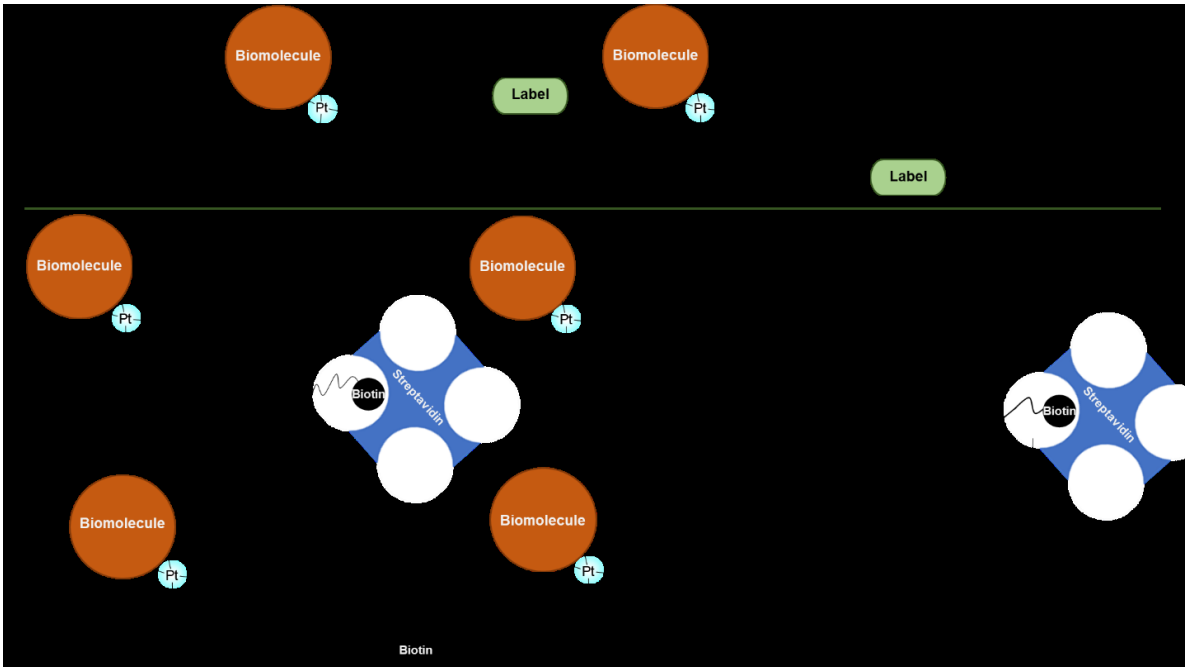

Figure 2. Affinity purification of Pt(II)-bound biomolecules by click chemistry. A. Pt(II)-bound biomolecules are labeled with an alkyne-containing molecule (fluorophore, biotin, oligonucleotide) by the click reaction. B. Scheme of direct biotin labeling using biotin alkyne with a PEG4 linker. Following capture by streptavidin beads, Pt-bound and biotinylated species are eluted by heating to 90C in urea or detergent-containing buffer. C. Scheme of DNA hybridization-based pull-down. Pt-bound species can be eluted at temperatures just above the melting temperature of the hybridized DNA.

Here, we demonstrate successful click ligation of a hexynyl-modified DNA oligonucleotide to azidoplatin-bound hairpin DNA or bovine serum albumin (BSA) followed by enrichment of the Pt(II)-bound species. Over the course of these studies, we also found

that Pt(II) adducts on BSA can form secondary crosslinks with DNA regardless of click ligation conditions and using non-functionalized cisplatin, suggesting that the Pt-BSA adducts are capable of forming an additional Pt-DNA bond and are perhaps initially monofunctional. Using a biotinylated DNA oligonucleotide we are able to specifically enrich for these Pt-protein adducts in the absence of click-modified Pt reagents. We also demonstrate transfer of Pt(II) from BSA to a DNA hairpin, further indicating that cisplatin that is already bound to serum albumin can react with DNA. Finally, we were interested in the properties of DNA crosslinked through Pt(II) to a serum protein. Serum stability assays show that DNA that is crosslinked to BSA by cisplatin is highly stable in the presence of serum nucleases.

2. Materials and Methods

2.1 Chemicals

Azidoplatin [22], 2-ADAPPt [21], and rhodamine B-alkyne [22] were synthesized as described previously. Cisplatin was purchased from Strem Chemicals (78-0450). Oligonucleotides were purchased from Integrated DNA Technologies; DNA hairpin (5'-TAT GGT ATT TTT ATA CCA TA-3'), hex-DNA (5'- /Hexynyl/TTT TTT TCT GTA GGC ACC ATC AAT -3'), bio-DNA (5'- /Biotin/TTT TTT TTT ATT GAT GGT GCC TAC AG -3'), nonspecific blocking oligonucleotide (block-DNA)
(5'-CAA GCA GAA GAC GGC ATA CGA GAT GTC ATG TAC TGG AGT TCA GAC GTG TG CTC TTC CG-3'). Lyophilized bovine serum albumin was purchased from Sigma Aldrich (A4612) and resuspended in milli-Q water to a concentration of 10 mg/mL. SYBR Gold DNA stain (S-11494) and Dynabeads MyOne streptavidin C1 magnetic beads (11205D) were purchased from Thermo Fisher Scientific.

2.2 Platination of DNA

Prior to platination, DNA hairpin was denatured for 5 min at 95°C followed by 30 min of slow cooling. 40 μM hairpin DNA was incubated with 120 μM azidoplatin in the presence of 100 mM $NaNO_3$, 10 mM phosphate buffer (pH 7.0) and 1 mM $Mg(NO_3)_2$ for 18 h at room temperature. DNA platination reactions were cleaned up by ethanol precipitation and resuspended in milli-Q water.

2.3 Click Reaction and DNA Hybridization

For the click reaction, 3 μM AzPt-treated hairpin DNA was reacted with 13 μM hex-DNA, 75 μM $CuSO_4$, 375 μM Tris(3-hydroxypropyltriazolyl-methyl)amine (THPTA), and 150 μM sodium ascorbate in 100 mM phosphate buffer (pH 7.0) for 2 h at 37°C with rotation. Modified DNA was then isolated by ethanol precipitation. 100 pmol of purified hex-DNA labeled hairpin DNA was hybridized to 100 pmol of bio-DNA by heating to 90°C for 5 min followed by slow cooling to room temperature.

2.4 Streptavidin pull-down of hybridized DNA

Dynabeads MyOne streptavidin C1 magnetic beads were washed and prepped according to manufacturer's instructions. To reduce nonspecific binding of DNA, a blocking step was performed prior to adding the click reaction products. For this purpose, 1 nmole of block-DNA in a volume equal to the click reaction volume was added to beads and incubated for 20 min at room temperature with rotation. Beads were washed three times with 1X bind and wash buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M $NaNO_3$). Hybridized DNA was then added to streptavidin-coated beads and allowed to bind for 18h overnight with rotation at 4°C. Following binding, beads were washed in 1X bind and wash buffer seven times with decreasing concentrations of $NaNO_3$ (1 M $NaNO_3$ to 100 μM $NaNO_3$). For elution of the

bound DNA, beads were resuspended in sodium citrate buffer (0.15 M NaCl, 0.015 M $Na_3C_6H_5O_7$, pH = 7.0) and heated to 95°C for 5 min. Eluate was collected and purified by ethanol precipitation prior to analysis by denaturing urea polyacrylamide gel electrophoresis (dPAGE)(20% 19:1 acrylamide:bisacrylamide). DNA was visualized in-gel by SYBR Gold staining.

2.5 Bovine Serum Albumin Platination Reactions

Fresh 5 mM solutions of AzPt, 2-ADAPPt, and cisplatin were prepared in milli-Q water. 1 mM bovine serum albumin was incubated with 1, 2, or 5 mM AzPt, 2-ADAPPt, or cisplatin for 18 h at room temperature in the presence of 10 mM Na_2PO_4 (pH 7.4) and 1 mM $Mg(NO_3)_2$. Excess Pt(II) was removed by Sephadex G-25 medium (GE Life Sciences, 17003301) in laboratory prepared spin columns (Thermo Fisher, 89868).

2.6 DNA Crosslinking with BSA

50 µM BSA that had been treated with 1, 2, or 5 molar equivalents of AzPt, 2-ADAPPt, or cisplatin was incubated with 250 µM 5' hex-DNA in milli-Q water for 1 h at room temperature. For DNA hairpin crosslinking experiments, 250 µM hairpin DNA was used. Samples were then resolved by dPAGE using a stacking gel (10% stacking/20% resolving). Gels were first stained with SYBR Gold and imaged using an AlphaImager system, then stained using coomassie brilliant blue.

2.7 Biotin Labeling and Pull Down of DNA-BSA Crosslinks

50 µM BSA that had been treated with 5 molar equivalents of cisplatin was incubated with 250 µM 5' biotin-modified DNA in milli-Q water for 1 h at room temperature. Reactions were then cleaned up by Sephadex G-25 medium spin columns. 10 µL of the BSA-DNA (half

reaction) was brought to 30 µL in PBS and added to 30 µL agarose streptavidin beads. Beads were incubated at 4°C overnight with rotation. Unbound protein was collected, beads were washed 5 times with PBS, and protein was eluted using 2% SDS loading buffer. The unbound fraction ("flow through") and elution were resolved by dPAGE. 10 µL of biotin-labeled BSA that was not incubated with streptavidin beads was used as "input." Gels were first stained with SYBR Gold and imaged using an AlphaImager system, then stained for protein using coomassie brilliant blue.

2.8 Pt(II) transfer from BSA to DNA

50 µM of BSA that had been treated with either 1 or 5 molar equivalents of cisplatin (as described above) was incubated with 50 µM hairpin DNA oligonucleotide for 1, 6, 24, and 48 h at 37°C. Following incubation for the indicated time periods, formamide was added to stop the reaction. To confirm that Pt(II) transfer was not due to inadequate removal of Pt(II) following BSA platination reactions, BSA treated with 1, 2, or 5 molar equivalents of cisplatin that had already been cleaned up by G-25 sephadex spin columns was further purified using Amicon 3 kDa MWCO centrifuge filtration units (Sigma Aldrich, Z677094). As any remaining cisplatin is not large enough to be retained by the filter, eluate was collected as it would contain any free cisplatin. BSA and cisplatin-bound BSA is retained by the filter and was collected as purified BSA. Following filtration, both eluate and purified BSA were adjusted to their original volumes with milli-Q water. As above, 50 µM hairpin DNA was incubated with either 50 µM unfiltered platinated BSA, 50 µM purified platinated BSA, or an equal volume of eluate. A single incubation time point of 24 h was collected and formamide added to stop the reaction. All samples were separated by dPAGE and gel was first stained with SYBR Gold followed by coomassie brilliant blue.

2.9 Click Reaction of DNA Crosslinked BSA with a Fluorophore

Pt(II)-bound BSA that had been crosslinked to hex-DNA (as described above) was reacted with 50 μM rhodamine B-alkyne, 125 μM $CuSO_4$, 500 μM THPTA, and 2.5 mM nmol sodium ascorbate for 1 h at room temperature. Reactions were then cleaned up by Sephadex G-25 medium in spin columns and eluent resolved by dPAGE. Rhodamine B-alkyne labeling was first imaged using an AlphaImager system, followed by staining with SYBR Gold and coomassie brilliant blue.

2.10 DNA extraction and shearing

MDA-MB-468 cells were grown to confluency in drug-free RPMI media supplemented with 10% fetal bovine serum. Cells were detatched, pelleted, and washed in 1x PBS. Cells were then lysed by incubating at 50°C for 1 h in lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, EDTA pH 8.0, 0.5% SDS) containing proteinase K. DNA was extracted from lysate by addition of 1 volume phenol:chloroform:isoamyl alcohol. Following ethanol precipitation, DNA was resuspended in milliQ water. DNA was sheared using a Covaris ME220 focused ultrasonicator set for 150 bp fragments. Sheared DNA was next separated on a 2% agarose gel, and DNA of ~150 bp was gel purified using the GeneJet gel extraction kit (Thermo Fisher). Final concentration of purified DNA was determined by Nanodrop.

2.11 Serum stability of DNA-BSA crosslinks

50 μM BSA that had been treated with 5 molar equivalents of cisplatin (as described above) was incubated with 50 μM hairpin DNA for 1 h at 37°C. For sheared cell DNA experiments, cisplatin treated BSA was incubated with 5 μM sheared DNA for 24 h at 37°C. Following crosslinking, fetal bovine serum (FBS, ThermoFisher) was added to a final concentration of 20%. An aliquot was immediately taken and formamide added as the “0 h” time point.

Subsequent aliquots were collected at indicated time points. Half of the serum digest reaction was resolved on a denaturing polyacrylamide stacking gel. Crosslinked DNA was stained by SYBR Gold and imaged using an AlphaImager system. Protein was visualized by coomassie staining. For hairpin DNA experiments, the resolving gel containing free DNA was stained by methylene blue, stacking gel was stained with SYBR Gold and coomassie. Band intensities of SYBR Gold stained DNA-BSA crosslinks and methylene blue stained free DNA were determined using GelAnalyzer (gelanalyzer.com) software. Percent DNA was calculated for DNA-BSA crosslinks and free DNA separately, using the 0h time point for normalization.

3. **Results and Discussion**

3.1 DNA oligonucleotide conjugation to AzPt-bound DNA hairpin

We have previously used azidoplatin (AzPt, Figure 1) for click ligation of fluorophores to DNA hairpins [20-22]. Here, we first wanted to confirm that we could also successfully label AzPt-bound DNA with a hexynyl-functionalized DNA oligonucleotide (hex-DNA). We first treated the DNA hairpin (HP) with azide-functionalized AzPt, and then performed the click reaction with hex-DNA. Analysis by dPAGE clearly shows that a click-functionalized oligonucleotide can attach to DNA platinated with a click-modified platinum reagent (Figure 3). We observe a shift in molecular weight following the click reaction as evidenced by the appearance of an extra, high molecular weight band in the sample containing the Cu(I) catalyst. Successful click-ligation of hex-DNA to AzPt-bound DNA is expected to approximately double the size of the molecules. A control reaction that lacks the Cu(I) catalyst shows little intensity for the click-ligated band, confirming that this interaction is mediated by the click reaction. Attaching two DNA strands together through a click-

enabled platinum reagent is a novel result and suggests success for the ensuing hybridization pull-down approach.

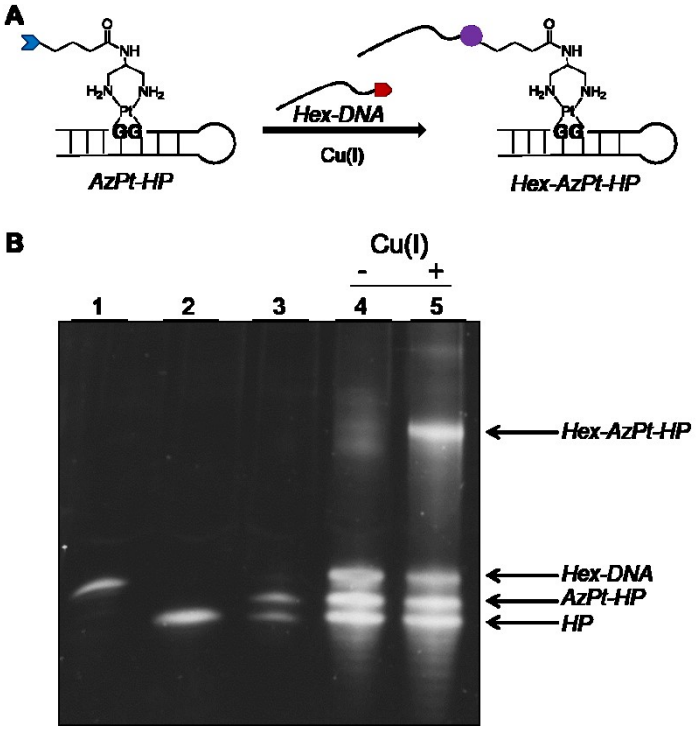

Figure 3. A. Reaction scheme showing click ligation of hex-DNA to AzPt-bound DNA HP. B. Successful click reaction between AzPt-HP and hex-DNA. Gel displays the successful click reaction between a platinated DNA strand (AzPt-HP) and a click-functionalized DNA strand (hex-DNA). Lanes are as follows: (1) Hex-DNA; (2) HP; (3) HP treated with AzPt (upper band is AzPt-HP); (4-5) (-) Cu control and (+) Cu click reaction between AzPt-HP and hex-DNA.

*3.2 Enrichment of AzPt-bound DNA hairpin using hybridization-based pull-down*After successfully click-ligating hex-DNA to AzPt-HP, we next tested whether we could isolate platinated DNA via hybridization-based streptavidin pull-down (Figure 4). Platinated DNA that had been click-conjugated with hex-DNA was hybridized to a biotinylated DNA oligonucleotide (bio-DNA) of a complementary sequence. The hybridized sample was incubated with streptavidin beads to allow capture of biotinylated DNA. Following isolation by centrifugation, elution was performed by heating above the hybridization temperature. Samples were taken from each step for analysis.

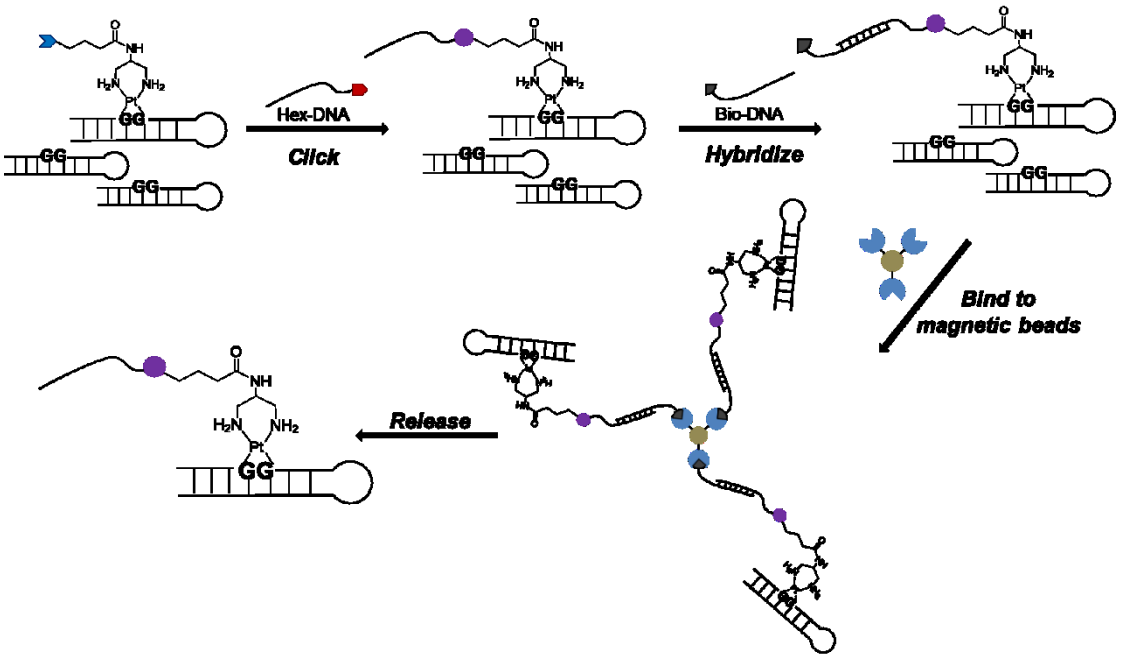

Figure 4. Workflow for isolation of AzPt-bound hairpin DNA. Platinated DNA is click-ligated to hex-DNA, which is then hybridized to biotinylated bio-DNA and isolated using streptavidin-coated magnetic beads. Elution at elevated temperatures releases the desired platinated molecule.

dPAGE analysis shows that this procedure effectively isolates platinated, clicked DNA from a complex mixture of DNA species (Figure 5). Comparison of eluted product (lane 6) with flow-through (lane 4) shows significant enrichment of the high molecular weight click product Hex-AzPt-HP over unclicked or unplatinated HP DNA. Significant levels of bio-DNA are also eluted, indicating that the temperature used for elution results in release of both hybridized hex-DNA and bio-DNA components. Of note, in this procedure, the beads were first incubated with non-biotinylated 'blocking DNA' to reduce nonspecific DNA-bead interactions. A very faint band above the click product in the elution lane is identifiable as a small amount of blocking DNA that is also released from the beads upon heating. Also observed is a low level of unplatinated, unclicked hex-DNA that is carried through in the procedure. In applying this procedure to identify novel platinated species, both of these known sequences could be easily removed in downstream analyses.

3.3 AzPt-treated BSA associated with hex-DNA independent of the click reaction

We are interested in using click-enabled Pt(II) compounds to identify interactions with proteins in addition to nucleic acids. Recent work reported successful identification of Pt(II)-bound proteins in AzPt-treated *Saccharomyces cerevisiae* using biotin-streptavidin pull-down and proteomics analysis, identifying 152 proteins that appear to be targeted by Pt(II) [15]. We wanted to test the efficiency of the new DNA hybridization strategy for Pt(II)-protein labeling. To accomplish this, we performed *in vitro* experiments using bovine serum albumin (BSA), a model protein that has been previously used to study serum protein-Pt(II) interactions [21,25,26].

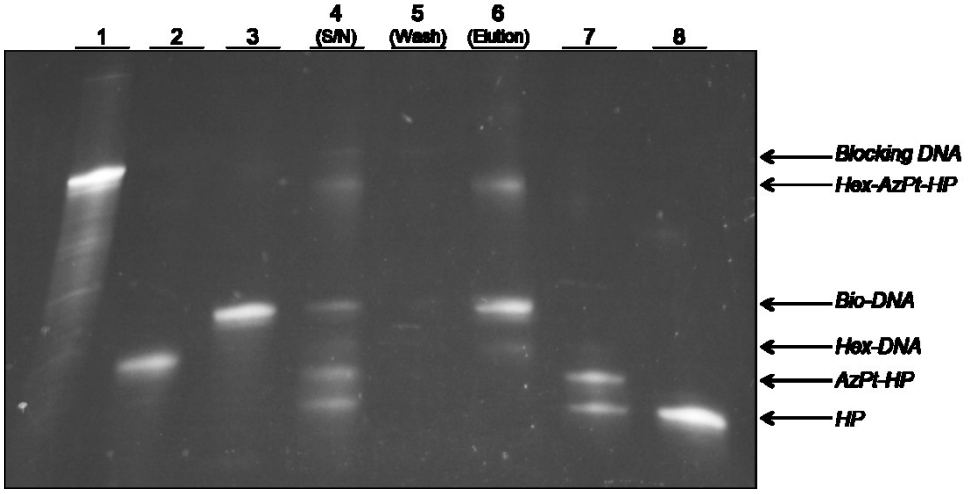

Figure 5. Hybridization-based pull-down is effective in enriching for Hex-AzPt-HP. Lanes are as follows: (1) Block-DNA; (2) Hex-DNA; (3) Bio-DNA; (4) Supernatant; (5) Wash 1; (6) Elution; (7) HP + AzaPt-HP (8) HP. Samples were separated by dPAGE and DNA stained by SYBR Gold fluorescence.

We first demonstrated that, as with the DNA substrate discussed above, AzPt-treated BSA could be click-ligated to hex-DNA. The reaction was monitored following separation by dPAGE and staining with either Coomassie or SYBR Gold to detect protein or DNA, respectively. Multiple BSA bands are observed in-gel, with monomeric BSA migrating the fastest, and slower migrating aggregates above. Covalent attachment of hex-DNA to BSA is indeed observed as bands that appear with both SYBR Gold and coomassie stain (Figure 6). Surprisingly, however, this interaction was not dependent on the click reaction, as control reactions that did not contain Cu(I) catalyst also showed association of the DNA oligonucleotide and BSA. Association between DNA and BSA was not observed in the absence of the Pt compound (see below). These results indicate a Pt(II)-mediated association of DNA and protein that is independent of click ligation.

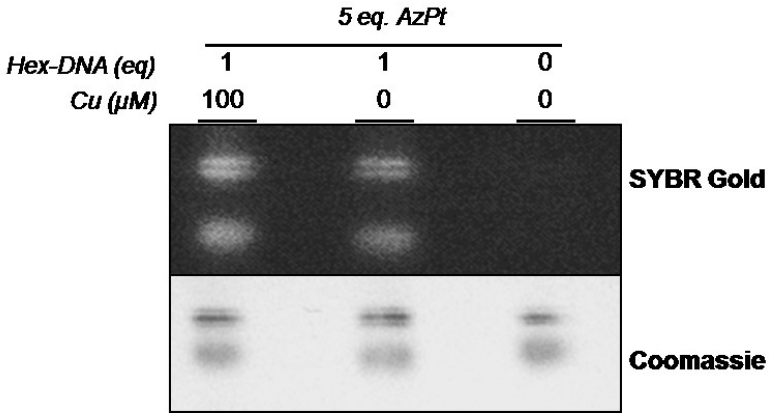

Figure 6. AzPt-treated BSA crosslinks Hex-DNA independent of the click reaction. dPAGE analysis of BSA treated with 5 eq. AzPt reacted with 1 eq. Hex-DNA and all click reactants or without the Cu catalyst. DNA was stained by SYBR Gold (top) followed by protein staining with coomassie (bottom). Association of AzPt-BSA with Hex-DNA is observed with and without the Cu catalyst. Lower band is BSA monomer, higher bands are likely aggregates.

Cisplatin and AzPt can form bifunctional adducts with ligands on biomolecules through loss of both chloride ligands. Cisplatin is also able to form monofunctional adducts wherein one labile Pt(II) ligand remains unsubstituted. Previous studies have predicted that cisplatin forms monofunctional adducts on serum albumin in addition to the more frequently reported bifunctional adducts [27,28]. We therefore hypothesized that AzPt may be forming monofunctional Pt(II) adducts on BSA, leaving an unsubstituted site on Pt(II) that is able to react with guanine or adenine on the DNA, effectively crosslinking the protein and DNA. This behavior has been observed before, as a monofunctional adduct on ubiquitin is able to crosslink to 5' guanosine monophosphate [29]. Pt(II) drugs have also previously been shown

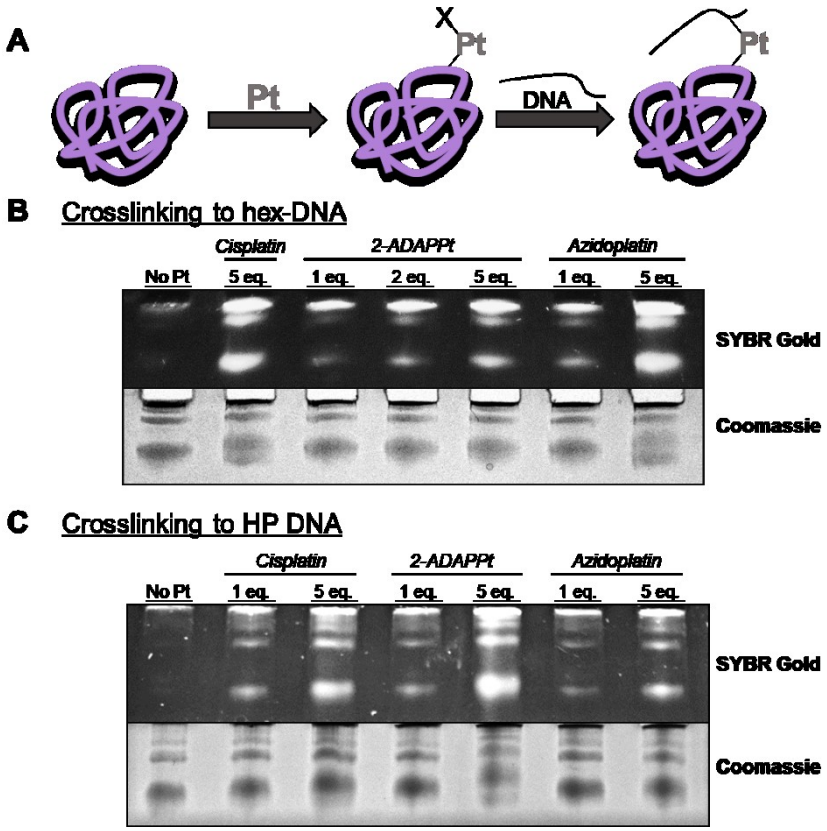

Figure 7. DNA-BSA crosslinking is observed with multiple Pt(II) compounds. A Scheme of reaction. BSA was first treated with cisplatin or an azide-modified Pt compound for 18h, then incubated with hex-DNA or HP DNA for 1h. dPAGE analysis of (B) hex-DNA and (C) HP DNA crosslinks to platinated BSA.. DNA was stained using SYBR Gold (top), and protein stained by Coomassie (bottom). No DNA co-migrates with untreated 'no-Pt' BSA, whereas DNA co-migrates with BSA treated with all three Pt(II) compounds.

to participate in DNA-protein crosslinking *in vitro* and in cells [30-34]. To further test this behavior, we compared DNA-protein crosslinking in azide-free cisplatin as well as both click-modified reagents, 2-ADAPPt and AzPt. We also tested the influence of the hexynyl moiety on DNA. The results (Figure 7) demonstrate that DNA-protein crosslinking occurs readily with cisplatin, and with unmodified DNA lacking a hexynyl modification.

We next tested whether we could perform Pt-mediated crosslinking to a biotin-modified DNA oligonucleotide and then enrich for Pt-protein species with streptavidin pull-down. To do this, we treated BSA with 5 molar equivalents of cisplatin followed by incubation of Pt(II)-treated BSA with bio-DNA, and subsequent streptavidin-bead pulldown. Pt-BSA that is crosslinked to bio-DNA is indeed bound and eluted from beads (Figure 8), whereas BSA incubated with the biotinylated oligonucleotide in the absence of cisplatin is not retained. Some Pt-BSA crosslinked with DNA is also observed in the flow-through, indicating incomplete binding to the streptavidin beads under these conditions.

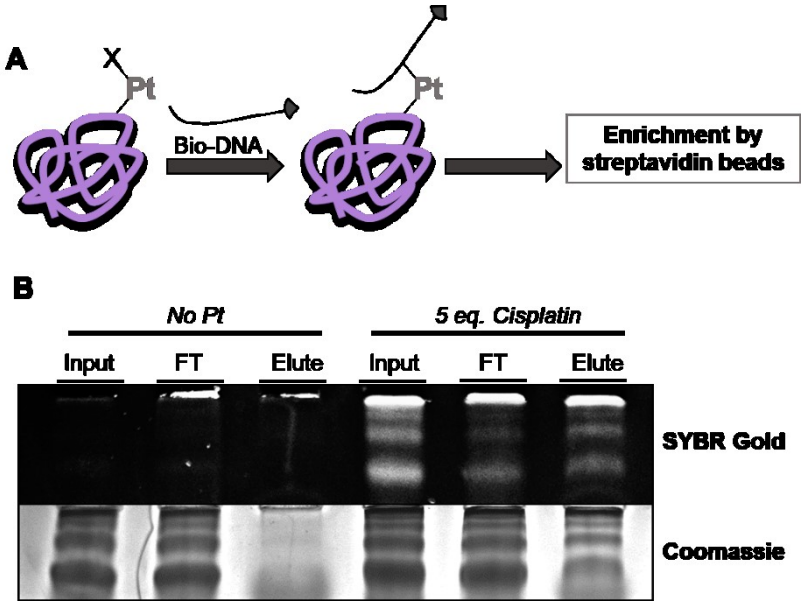

Figure 8. Biotin-streptavidin pull-down of BSA-Pt-DNA crosslinks. A. Scheme of reaction. BSA was treated with 5 eq. of cisplatin for 18h, then incubated with bio-DNA for 1 h. BSA-Pt-DNA crosslinks were enriched for using streptavidin coated beads. B. dPAGE analysis shows unbound protein from the beads was collected as flow through (FT), and bound protein in the elution. DNA was stained by SYBR Gold and protein was stained by coomassie.

3.4 Transfer of Pt(II) from BSA to DNA

To learn more about the properties of DNA-Pt-BSA crosslinked species, we tested the stability of crosslinks over an extended incubation time. To do this, BSA was pre-treated with either 1 or 5 molar equivalents of cisplatin and excess cisplatin removed. Then, 1 molar equivalent of DNA hairpin was incubated with the cisplatin-BSA samples and the mixture analyzed at various time points. dPAGE analysis showing only DNA species as stained by SYBR Gold reveals the expected appearance of DNA-bound BSA as well as free HP DNA. Interestingly, over time an additional band just above the free DNA hairpin appears in samples incubated with cisplatin-treated BSA (Figure 9). This band migrates similarly to cisplatin-bound hairpin DNA, indicating that the hairpin DNA is being platinated during the extended incubation. As the only source of Pt(II) in the solution is already bound to BSA, this suggests that Pt(II) bound to BSA is being transferred from the protein to the hairpin DNA. This result is most evident for BSA treated with 5 molar equivalents of cisplatin, with the Pt-DNA band appearing after 6 h of incubation and growing in intensity by 24 h. The amount of Pt(II) bound to BSA appears to influence Pt(II) transfer, as BSA treated with only 1 molar equivalent of cisplatin shows only a very faint band corresponding to Pt(II)-bound DNA after 24h of incubation.

We performed the same experiment using our click-enabled Pt(II) compound 2-ADAPPt with post-treatment fluorescent labeling to confirm that the shifted band was indeed due to Pt(II) binding (Supplemental Information). Additional experiments also confirmed that

the transfer of Pt from protein to DNA was not due to free cisplatin that had not been removed following BSA platination reactions (Supplemental Information).

Intermolecular transfer of Pt(II) from protein to DNA has only been described a handful of times. Experiments using a monofunctional [Pt(diethylenetriamine)Cl]$^+$ compound demonstrated exchange of free methionine for free guanosine 5'-monophosphate [35] as well as the dinucleotide d(GpG) [36]. This exchange of methionine for 5'-GMP was also shown to take place with methionine reacted with cisplatin [36]. On a macromolecule scale, Pinato et al. observed Pt(II) transfer to a fluorescein-labeled DNA oligonucleotide from bovine α-lactalbumin that had been treated with a transplatin derivative [37]. To the authors' knowledge, this is the first report of cisplatin transfer from serum albumin to DNA.

This finding has biological significance as it indicates that following binding to serum albumin, some Pt(II) is still reactive towards DNA. Many reports have found that a significant amount of platinum derived from intravenously-administered cisplatin is found in the blood serum, most likely due to binding with abundant serum proteins. Some suggest that one day following treatment, up to 90% of cisplatin-derived Pt(II) is bound to proteins in serum from treated patients [38,39]. It has been hypothesized that binding of cisplatin-derived

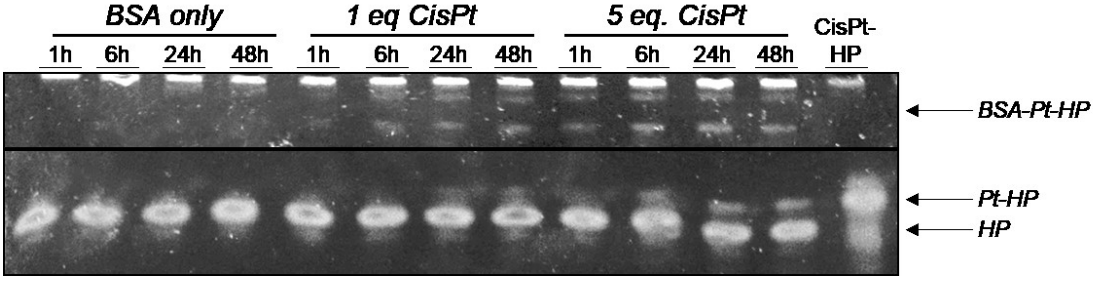

Figure 9. Transfer of Pt(II) from cisplatin-treated BSA to DNA hairpin. BSA treated with 1 or 5 molar equivalents was incubated with 1 molar equivalent of DNA hairpin for up to 24 h. DNA was stained by SYBR Gold. Highest bands in fluorescent image represent DNA crosslinked to BSA by cisplatin. More intense low band represent free DNA hairpin, and lighter band just above represents Pt-bound DNA as confirmed by DNA hairpin (treated with 1 eq cisplatin) size control in final lane. Shown are upper and lower portions of gel; full gel can be found in Appendix A.

Pt(II) species to serum albumin may essentially deactivate the drug, sequestering it from reaching the cell to exert its cytotoxic effect [40,41]. However, other research has found that patients with decreased levels of serum albumin tend to respond poorly to cisplatin treatment [42,43], indicating that the protein may aid in delivering cisplatin to cells. Additionally, delivery of cisplatin pre-bound to serum albumin alleviated nephrotoxicity in a patient with head and neck carcinoma [44]. This ligand exchange from protein to DNA further supports the hypothesis that serum albumin may act as a more active drug reservoir [42,44-45].

3.5 Stability of DNA crosslinked to BSA by cisplatin in the presence of serum nucleases

We next set out to investigate the stability of DNA exposed to serum nucleases while crosslinked to BSA. There has been much interest in using serum albumin as a carrier for nucleic acid delivery to cells, as free nucleic acids are prone to nuclease degradation in the serum [46-48]. A recent report found dendritic alkyl chains conjugated to DNA for binding to human serum albumin significantly increased stability in serum [49]. We therefore set out to test if DNA crosslinked to BSA by cisplatin had similar enhanced serum stability. To do this, hairpin DNA was first crosslinked to cisplatin-treated BSA for 1 h. Fetal bovine serum was next added to the reaction, and aliquots were removed at time points up to 24 h. Stability of the crosslinked and free DNA were observed by dPAGE analysis (Figure 10A). Free hairpin DNA is quickly degraded, and full disappearance of the HP DNA band occurs by 24 h. The half-life of free hairpin DNA is approximately 4.5 h, which is slightly longer than reported half-lives for DNA oligonucleotides of similar lengths which range from 10 min to over 2 h [49-51]. This may be due to the stable hairpin structure formed by the hairpin DNA which can influence its susceptibility to nuclease degradation [52-54]. Interestingly, however, DNA that is crosslinked to BSA has much greater stability over this timeframe, with loss of only

20% by the 24 h timepoint based on SYBR Gold intensity analysis of the HP DNA-Pt-BSA bands.

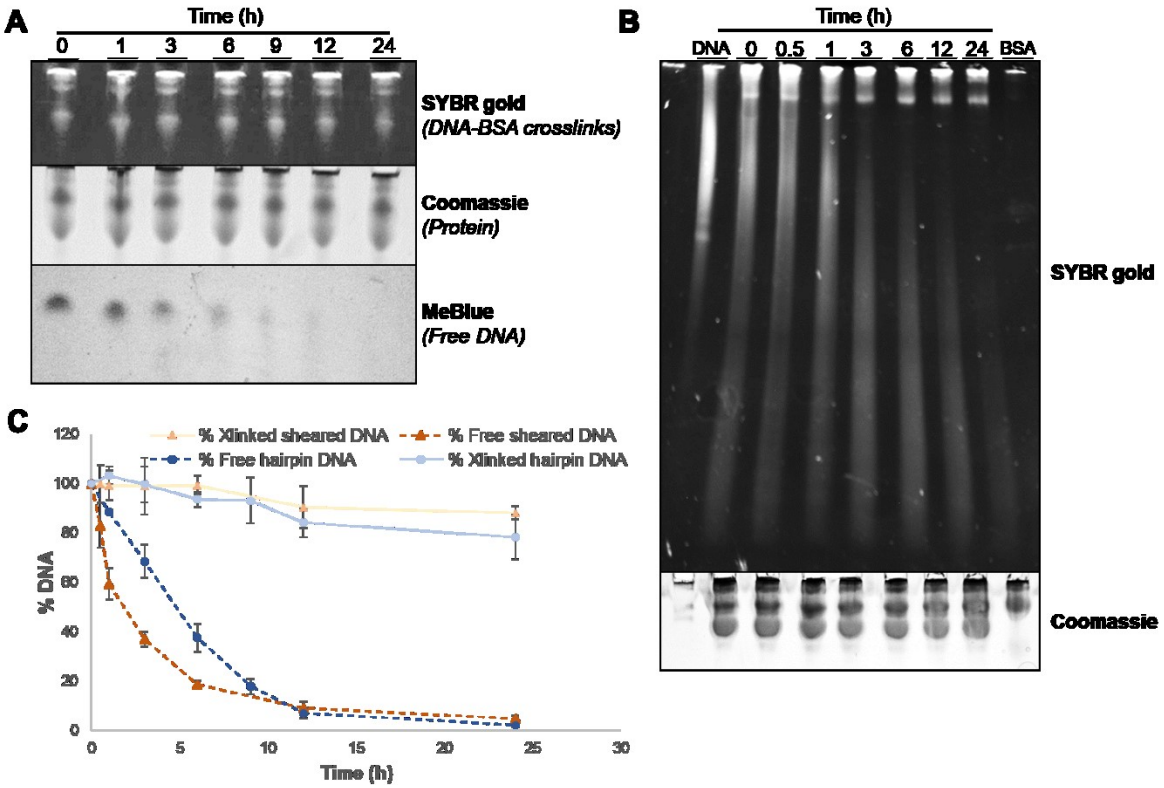

Figure 10. DNA-Pt-BSA crosslinks are stable in the presence of serum nucleases. BSA pre-treated with 5 molar equivalents of cisplatin was incubated with (A) hairpin DNA for 1 h or (B) ~150 nt fragmented DNA from MDA-MB-468 cells, after which fetal bovine serum was added to the reaction. DNA crosslinked to BSA was detected by SYBR Gold staining, and protein stained for by Coomassie. For DNA hairpin experiments, methylene blue was used to stain for free DNA as the DNA concentration was too high for quantitative SYBR Gold staining. (C) Band intensity was used for quantification, and % DNA reported relative to the 0 h time point

The hairpin DNA is a small oligonucleotide of 20 nucleotides. We were interested in testing whether this protective effect also occurs with longer DNA sequences. It is well established that free DNA can be found circulating in serum, and these circulating DNAs are found at a significantly higher concentration in serum from cancer patients [55]. Because of this, circulating tumor DNAs (ctDNA) are being investigated as a potential biomarker for many cancers [56]. While source and biological function of ctDNA is still unclear, the average length of ctDNA is known to be around 150 bp as nucleosomes or naked DNA and

likely originates from apoptotic cleavage of cellular DNA [57,58]. Half-lives of ctDNA in the bloodstream are reported to be 1.5–2.5 h [56,59].We decided to use this as a biologically relevant size for DNA that might be found in serum. For these experiments, DNA extracted from the triple negative breast cancer cell line MDA-MB-468 was fragmented to approximately 150 bp. Although the fragmented DNA represents a variety of sequences, robust crosslinking with Pt-BSA is observed as an almost complete depletion of free DNA when incubated with cisplatin-treated BSA (Figure 10B). Analysis of the BSA-Pt-DNA samples upon 24 hr incubation in serum reveals a nearly complete loss of non-crosslinked DNA with an approximate half-life of 2 h, but significant nuclease protection of DNA bound to BSA with over 80% remaining over the same timeframe. It is possible that exonucleases digest the ends of crosslinked DNA, but if this is occurring we predict it to be a relatively small amount based on the less than 20% loss in DNA staining over the timecourse of the experiment.

Taken together, these results indicate that BSA crosslinking mediated by Pt compounds can significantly increase stability of longer DNA sequences in serum. Analysis of DNA-protein crosslinks in cisplatin treated cells [34] has shown evidence of DNA crosslinking to histone proteins following treatment. As protein-DNA crosslinking appears to protect bound DNA from serum nucleases, it would be interesting to investigate whether protein-DNA crosslinks influence stability of ctDNA. In accordance with other reports investigating oligonucleotide stability in serum [49-51,54,60], these experiments were performed at a pH of 7.4 using fetal bovine serum. It should be noted, however, that the slightly acidic tumor microenvironment of approximately pH 6.5-6.9 [61] may influence nuclease activity. As the major serum nuclease, DNase I [62], is reported to have optimal activity at pH 5.5-7.5 [63,64], the effect on nuclease degradation of oligonucleotides is likely to be minor.

4. **Conclusions**

We have demonstrated the ability to attach a DNA oligonucleotide to either a DNA or protein substrate treated with a clickable Pt reagent and have used this tag to isolate Pt(II)-bound biomolecules. This methodology can be applied to biological samples, for example in tagging and isolating Pt(II)-bound DNA from treated cancer cells.

We also discovered that BSA treated with bifunctional Pt(II) reagents becomes crosslinked to DNA in a Pt-dependent reaction. It is presumed that this occurs through a Pt-BSA adduct that retains one more labile Pt ligand, allowing for additional coordination of a DNA nucleobase. Further studies are necessary to identify the species responsible for this crosslinking. It can be difficult to distinguish the mode of Pt(II) binding using techniques such as mass spectrometry, as treatment of samples post-binding sometimes perturbs the Pt(II) complex causing loss of ligands [65,28]. It is possible that the method described here can select for monofunctional Pt-protein adducts. Using these methods upstream of other analytical techniques such as mass spectrometry can help deconvolute analysis, providing more confident assignments of Pt(II) binding sites and their mode of binding on serum albumin and other proteins.

We also investigated the properties of DNA-Pt-BSA crosslinks with respect to Pt transfer and protection of DNA in the presence of serum nucleases. A fraction of Pt(II) that is bound to BSA can transfer to free DNA, supporting hypotheses that serum albumin may act as a Pt(II) reservoir [42,44].

The amount of Pt(II) bound to BSA also appears to influence the transfer of Pt(II) to DNA, as evidenced by increased DNA hairpin platination in samples containing BSA that had been treated with higher equivalents of cisplatin. We hypothesize that Pt(II) might be binding more stable sites initially, such as Cys34 [26], while higher concentrations of cisplatin allow for adduct formation at sites that may be more prone to ligand exchange.

While we observe Pt(II) transfer from BSA to DNA, it appears to be a relatively small amount as a majority of the DNA remains crosslinked to BSA following a 48 h incubation.

We observed that DNA crosslinked to BSA is protected from nuclease degradation, and this protection is observed for small 20 nucleotide hairpin DNA as well as 150 bp fragmented cellular DNA. The latter size range is of interest, as it is representative of the average length of circulating tumor DNAs [57]. Increased serum stability of serum albumin-bound DNA could have further applications for the conjugation of nucleic acids to serum albumin for delivery. Additionally, these data indicate that DNA-protein crosslinks formed by Pt(II) increase intactness/stability of the DNA, which may aid in their detection.

Acknowledgements

We wish to thank Dr. Jonathan White, Dr. Alan Moghaddam, and Regina Wirth for their previous work synthesizing and characterizing the azide-modified Pt(II) compounds used in this study. Funding from the National Science Foundation (CHE-1413677 and CHE-1710721, V.J.D.), the American Heart Association (15PRE22820022, R.M.C.), the National Institutes of Health (T32-GM007759, K.I.J.P), the UO Presidential Undergraduate Research Scholars Program (A.H.), and the University of Oregon are gratefully acknowledged.

Appendix A. Supplementary data containing additional figures can be found online.

References

1) M. Schenone, V. Dančík, B.K. Wagner, P.A. Clemons, Nat. Chem. Biol. 9 (2013) 232-240, doi.org/10.1038/nchembio.1199
2) D.C. Swinney, J. Anthony, Nat. Rev. Drug Discov. 10 (2011) 507–519, doi.org/10.1038/nrd3480
3) T.J. Mitchison, Mol. Biol. Cell, 23 (2012) 1–6, doi.org/10.1091/mbc.E10-04-0335
4) B. Rosenberg, L. Van Camp, T. Krigas, Nature 205 (1965) 698–699, doi.org/10.1038/205698a0
5) L. Kelland, Nat. Rev. Cancer, 7 (2007) 573–584, doi.org/10.1038/nrc2167
6) E.R. Jamieson, S.J. Lippard, Chem. Rev. 99 (1999) 2467–2498, doi.org/10.1021/cr980421n
7) X. Shu, X. Xiong, J. Song, C. He, C. Yi, Angew. Chem. Int. Ed. Engl. 7 (2016) 14246–14249, doi.org/10.1002/anie.201607380
8) J. Hu, J.D. Lieb, A. Sancar, S. Adar, Proc. Natl. Acad. Sci. U.S.A. 113 (2016). 11507–11512, doi.org/10.1073/pnas.1614430113
9) K. Rijal, C.S. Chow, Chem. Commun. 0 (2008) 107–109, doi.org/10.1039/B816633A
10) A.A. Hostetter, M.F. Osborn, V.J. DeRose, ACS Chem. Biol. 7 (2012) 218–225, doi.org/10.1021/cb200279p
11) M.F. Osborn, J.D. White, M.M. Haley, V.J. DeRose, ACS Chem. Biol. 9 (2014) 2404–2411, doi.org/10.1021/cb500395z
12) S.V. Melnikov, D. Söll, T.A. Steitz, Y.S. Polikanov, Nucleic Acids Res. 44 (2016) 4978–4987, doi.org/10.1093/nar/gkw246
13) K. Plakos, V.J. DeRose, Chem. Commun. 53 (2017) 12746–12749, doi.org/10.1039/C7CC06708A

14) A. Casini, J. Reedijk, Chem. Sci. 3 (2012) 3135-3144, doi.org/10.1039/c2sc20627g

15) R.M. Cunningham, V.J. DeRose, ACS Chem. Biol. 12 (2017) 2737–2745, doi.org/10.1021/acschembio.7b00553

16) A. Mandic, J. Hansson, S. Linder, M.C. Shoshan, J. Biol. Chem. 278 (2003) 9100–9106, doi.org/10.1074/jbc.M210284200

17) F. Yu, J. Megyesi, P.M. Price, Am. J. Physiol. Renal Physiol. 295 (2008) F44-52, doi.org/10.1152/ajprenal.00593.2007

18) J.C. Jagodinsky, A, Sulima, Y. Cao, J.E. Poprawski, B.N. Blackman, J.R. Lloyd, R.E. Swenson, M.M. Gottesman, M.D. Hall, J. Biol. Inorg. Chem. 20 (2015) 1081–1095, doi.org/10.1007/s00775-015-1290-2

19) J.D. White, M.M. Haley, V.J. DeRose, Acc. Chem. Res. 49 (2016) 56–66, doi.org/10.1021/acs.accounts.5b00322

20) J.D. White, M.F. Osborn, A.D. Moghaddam, L.E. Guzman, M.M. Haley, V.J. DeRose, J. Am. Chem. Soc. 135 (2013) 11680–11683, doi.org/10.1021/ja402453k

21) A.D. Moghaddam, J.D. White, R.M. Cunningham, A.N. Loes, M.M Haley, V.J. DeRose, Dalton Trans. 44 (2015) 3536–3539, doi.org/10.1039/C4DT02649G

22) R. Wirth, J.D. White, A.D. Moghaddam, A.L. Ginzburg, L.N. Zakharov, M.M. Haley, V.J. DeRose, J. Am. Chem. Soc. 137 (2015) 15169–15175, doi.org/10.1021/jacs.5b09108

23) S.S. Oh, K. Plakos, X. Lou, Y. Xiao, H.T. Soh, Proc. Natl. Acad. Sci. U.S.A. 107 (2010) 14053–14058, doi.org/10.1073/pnas.1009172107

24) S.I. Sato, Y. Kwon, S. Kamisuki, N. Srivastava, Q. Mao, Y. Kawazoe, M. Uesugi, *J. Am. Chem. Soc. 129* (2007) 873–880, doi.org/10.1021/ja0655643

25) A.M. Krause-Heuer, W.S. Price, J.R. Aldrich-Wright, J. Chem. Biol. 5 (2012) 105–113, doi.org/10.1007/s12154-012-0074-1

26) A. Sugii, K. Nishimura, K. Harada, M. Nakayama, S. Masuda, Chem. Pharm. Bull. 39 (1991) 408–410, doi.org/10.1248/cpb.39.408

27) A.I. Ivanov, J. Christodoulou, J.A. Parkinson, K.J. Barnham, A. Tucker, J. Woodrow, P.J. Sadler, J. Biol. Chem. 273 (1998) 14721–14730, doi.org/ 10.1074/jbc.273.24.14721

28) J. Will, D.A. Wolters, W.S. Sheldrick, ChemMedChem 3 (2008) 1696–1707, doi.org/10.1002/cmdc.200800151

29) T. Peleg-Shulman, Y. Najajreh, D. Gibson, J. Inorg. Biochem. 91 (2002) 306–311, doi.org/10.1016/S0162-0134(02)00362-8

30) L.A. Zwelling, T. Anderson, K.W. Kohn, Cancer Res. 39 (1979) 365–369

31) Z. Banjar, L. Hnilica, R. Briggs, J. Stein, G. Stein, Biochemistry 23 (1984) 508–512

32) R. Olinski, A. Wedrychowski, W.N. Schmidt, R.C. Briggs, L.S. Hnilica, Cancer Res. 47 (1987) 201–205

33) K. Woźniak, Z. Walter, Naturforsch. C: J. Biosci. (2000) 55, 731–736, doi.org/ 10.1515/znc-2000-9-1010

34) X. Ming, A. Groehler, E.D. Michaelson-Richie, P.W. Villalta, C. Campbell, N.Y. Tretyakova, Chem. Res. Toxicol. 30 (2017) 980–995, doi.org/0.1021/acs.chemrestox.6b00389

35) K.J. Barnham, M.I. Djuran, P. del Socorro Murdoch, J.D. Ranford, P.J. Sadler, J. Chem. Soc. Dalton Trans. 0 (1995) 3721-3726, doi.org/10.1039/DT9950003721

36) S.S.G.E. van Boom, B.W.Chen, J.M. Teuben, J. Reedijk, Inorg. Chem. 38 (1999) 1450-1455, doi.org/10.1021/IC981086O

37) O. Pinato, C. Musetti, N.P. Farrell, C. Sissi, J. Inorg. Biochem. 122 (2013) 27–37, doi.org/10.1016/j.jinorgbio.2013.01.007

38) J.J. Gullo, C.L. Litterst, P.J. Maguire, B.I. Sikic, D.F. Hoth, P.V. Woolley, Cancer Chemotherapy and Pharmacology, 5(1980). 21–26. https://doi.org/10.1007/BF00578558

39) J.F. Neault, H.A. Tajmir-Riahi, Biochim. Biophys. Acta 1384 (1998) 153–159, doi.org/10.1016/S0167-4838(98)00011-9

40) W.C. Cole, W. Wolf, Chem-Biol. Interact. 30 (1980) 223–235, doi.org/10.1016/0009-2797(80)90128-3

41) K. Takahashi, T. Seki, K. Nishikawa, S. Minamide, J. Cancer Res. 76 (1985) 68–74, doi.org/10.20772/cancersci1985.76.1_68

42) J. Holding, W. Lindup, D. Bowdler, M. Siodlak, P. Stell, Br. J. Clin. Pharmacol. 32 (1991) 173–179, doi.org/10.1111/j.1365-2125.1991.tb03878.x

43) E. Espinosa, J. Feliu, P. Zamora, M.G. Barón, J.J. Sánchez, A. Ordónez, J. Espinosa, Lung Cancer 12 (1995) 67–76, doi.org/10.1016/0169-5002(95)00407-R

44) G.C.M. Vreeburg, P.M. Stell, J.D. Holding, W.E. Lindup, J. Laryngol. Otol. 106 (1992) 832–833, doi.org/10.1017/S0022215100121012

45) J.D. Holding, W.E. Lindup, C. Laer, G.C.M. Vreeburg, V. Schilling, J. Wilson, P.M. Stell, Br. J. Clin. Pharmacol. 33 (1992) 75–81, doi.org/10.1111/j.1365-2125.1992.tb04003.x

46) R.A. Cox, M. Gokcen, Life Sci. 19 (1976) 1609–1614, doi.org/10.1016/0024-3205(76)90108-9

47) E. Wickstrom, J. Biochem. Biophys. Methods 13 (1986) 97–102, doi.org/10.1016/0165-022X(86)90021-7

48) P.S. Eder, R.J. DeVine, J.M. Dagle, J.A. Walder, (1991). Antisense Res. Dev. 1 (2) 141–151, doi.org/10.1089/ard.1991.1.141

49) A. Lacroix, T.G.W. Edwardson, M.A. Hancock, M.D. Dore, H.F. Sleiman, J. Am. Chem. Soc. 139 (2017) 7355–7362, doi.org/10.1021/jacs.7b02917

50) A. Lakshmikuttyamma, Y. Sun, B. Lu, A.S. Undieh, S.A. Shoyele, Mol. Pharmaceutics 11 (2014) 4415-4424, doi.org/10.1021/mp500525p

51) S.N. Barnaby, A. Lee, C.A. Mirkin, Proc. Natl. Acad. Sci. 111 (2014) 9739-9744, doi.org/10.1073/pnas.1409431111

52) I. Hirao, G. Kawai, S. Yoshizawa, Y. Nishimura, Y. Ishido, K. Watanabe, K. Miura, Nucl. Acids Res. 22 (1994) 576-582

53) J. Fern, R. Schulman, ACS Synth. Biol. 6 (2017) 1774-1783, doi.org/10.1021/acssynbio.7b00105

54) A. Abdelgany, M. Wood, D. Beeson, J. Gene Med. 9 (2007) 727-738, doi.org/10.1002/jgm.1061

55) S.A. Leon, B. Shapiro, D.M. Sklaroff, M.J. Yaros, Cancer Res. 37 (1977) 646–650

56) H. Schwarzenbach, D.S.B. Hoon, K. Pantel, Nature Rev. Cancer 11 (2011) 426–437, doi.org/10.1038/nrc3066

57) H.R. Underhill, J.O. Kitzman, S. Hellwig, N.C. Welker, R. Daza, D.N. Baker, K.M. Gligorich, R.C. Rostomily, M.P. Bronner, J. Shendure, PLOS Genetics 12 (2016) e1006162, doi.org/10.1371/journal.pgen.1006162

58) S. Holdenrieder, D. Nagel, A. Schalhorn, V. Heinemann, R. Wilkowski, J. von Pawel, H. Raith, K. Feldmann, A.E. Kremer, S. Müller, S. Geiger, G.F. Hamann, D. Seidel, P. Stieber, Ann. N. Y. Acad. Sci. 1137 (2008) 180–189, doi.org/10.1196/annals.1448.012

59) F. Diehl, K. Schmidt, M.A. Choti, K. Romans, S. Goodman, M. Li, K. Thorton, N. Agrawal, L. Sokoll, S.A. Szabo, K.W. Kinzler, B. Vogelstein, L.A. Diaz Jr., Nat. Med. 14 (2008) 985-990, doi.org/10.1038/nm.1789

60) K. Nagahama, K. Iseda, D. Kawano, J. Kawakami, Biochimie 137 (2017) 124-131, doi.org/10.1016/j.biochi.2017.03.003

61) V. Estrella, T. Chen, M. Lloyd, J. Wojtkowiak, H.H. Cornnell, A. Ibrahim-Hashim, K. Bailey, Y. Balagurunathan, J.M. Rothberg, B.F. Sloane, J. Johnson, R.A. Gatenby, R.J. Gillies, Cancer Res. 73 (2013) 1524-1535, doi.org/0.1158/0008-5472.CAN-12-2796

62) M. von Köckritz-Blickwede, O.A. Chow, V. Nizet, Blood 114 (2009) 5245-5246, doi.org/10.1182/blood-2009-08-240713

63) S. Shak, D.J. Capon, R. Hellmiss, S.A. Marsters, C.L. Baker, Proc. Natl. Acad. Sci. 87 (1990) 9188-9192

64) D. Shiokawa, S. Tanuma, Biochemistry 40 (2001) 143-152 doi.org/ 10.1021/bi001041a

65) E. Moreno-Gordaliza, B. Cañas, M.A. Palacios, M.M. Gómez-Gómez, Anal. Chem. 81 (2009) 3507–3516, doi.org/10.1021/ac900046v