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Platinum(II) as Bifunctional Linker in Antibody–Drug Conjugate Formation: Coupling of a 4-Nitrobenzo-2-oxa-1,3-diazole Fluorophore to Trastuzumab as a Model

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The potential of platinum(II) as a bifunctional linker in the coordination of small molecules, such as imaging agents or (cytotoxic) drugs, to monoclonal antibodies (mAbs) was investigated with a 4-nitrobenzo-2-oxa-1,3-diazole (NBD) fluorophore and trastuzumab (Herceptin™) as a model antibody. The effect of ligand and reaction conditions on conjugation efficiency was explored for [Pt(en)(L-NBD)Cl](NO_3) (en = ethylenediamine), with L = *N*-heteroaromatic, *N*-alkyl amine, or thioether. Conjugation proceeded most efficiently at pH 8.0 in the presence of $NaClO_4$ or Na_2SO_4 in tricine or HEPES buffer. Reaction of N-coordinated complexes (20 equiv) with trastuzumab at 37 °C for 2 h, followed by removal of weakly bound complexes with excess thiourea, afforded conjugates with an NBD/mAb ratio of 1.5–2.9 that were stable in phosphate-buffered saline at room temperature for at least 48 h. In contrast, thioether-coordinated complexes afforded unstable conjugates. Finally, surface plasmon resonance analysis showed no loss in binding affinity of trastuzumab after conjugation.

Introduction

The coordination of platinum to DNA is an area of intense research that has allowed the development of valuable platinum-based chemotherapeutics.[1] Besides DNA, platinum also readily coordinates to other biomolecules, such as proteins.[2] Although platinum–protein binding has been studied extensively, this work is largely focused on the study of platinum–protein binding in cisplatin chemotherapy, where the uncontrolled binding of platinum(II) to proteins is believed to cause dose-limiting toxicity.[3] In this respect, the unique protein binding properties of platinum(II) still remain largely underexploited. The bivalent nature of cisplatin-type complexes, combined with the protein binding capacity of platinum, opens up the intriguing option to use these complexes as linkers in bioconjugation reactions. The complex [Pt(en)Cl_2] has been used in this context for the conjugation of kinase inhibitors to albumin and lysozyme in hepatic and renal targeting as well as targeting to activated endothelium.[4] Extension of this methodology to the preparation of antibody–drug conjugates (ADCs), for selectively targeting diagnostic or therapeutic agents to tumors or other diseases, would offer potential advantages over conventional methods (Figure 1).[5] More specifically, this approach would, in principle, allow the coordination of small molecules via a wide variety of coordinating groups, including nonconventional functionalities such as *N*-heteroaryl groups and S-donors, such as thioethers. Moreover, the introduction of a charged platinum complex can improve the aqueous solubility of organic compounds, especially at neutral pH, potentially obviating the need for organic co-solvent in subsequent conjugation reactions. Exposure of antibodies to organic solvents can induce aggregation, and is therefore typically avoid-

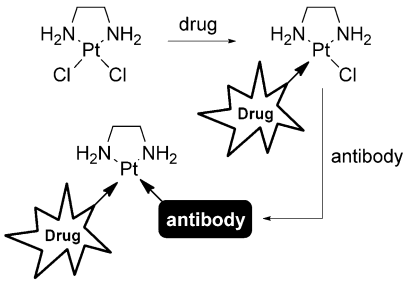

Figure 1. Platinum(II)-mediated conjugation of a drug (diagnostic or therapeutic compound) to an antibody.

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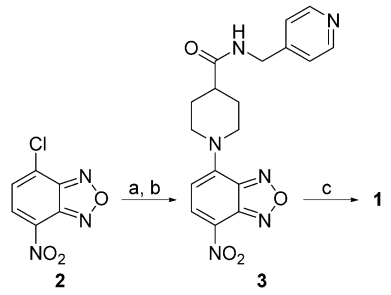
ed. Apart from these differences, platinum(II) is also expected to coordinate to a unique subset of amino acids. Extensive research in this area has shown that cysteine, methionine, and histidine are the most likely coordination sites for platinum in proteins.[6] Results from model systems[7] and density functional theory calculations[8] indicate that binding of platinum(II) to methionine is kinetically controlled, whereas binding to histidine is thermodynamically controlled. When performed under thermodynamic conditions, binding of platinum(II) to proteins also offers the prospect of high selectivity. X-ray diffraction analysis studies by Calderone et al. on the binding of cisplatin to bovine erythrocyte copper–zinc superoxide dismutase, for instance, revealed that cisplatin exclusively binds to His19.[9]



Scheme 1. Synthesis of complex **1**. *Reagents and conditions*: a) 4-piperidinecarboxylic acid, K_2CO_3, MeOH, 0 °C, 15 min, then RT, 2 h, 92%; b) 4-(aminomethyl)pyridine, BOP, Et_3N, MeCN/CH_2Cl_2, RT, 5 h, 99%; c) [Pt(en)Cl_2], $AgNO_3$, DMF, RT, 40 h, 26%. BOP = (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; en = ethylenediamine.

Results and Discussion

Synthesis and characterization of complex 1

Screening of reaction conditions was performed with complex **1**, in which the NBD group is coordinated to platinum through a 4-substituted pyridine (Figure 2). Although direct coupling of 4-(aminomethyl)pyridine to the NBD group would be most

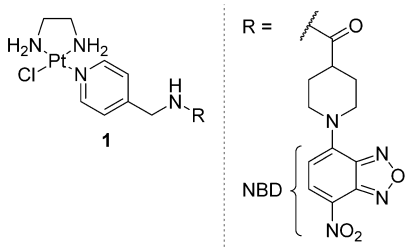


Figure 2. Structure of complex **1**.

straightforward, we chose to use a short spacer to prevent potential intramolecular coordination of platinum to the NBD group.[10]

Complex **1** was synthesized in three steps from NBD-Cl (**2**) (Scheme 1). After introduction of the pyridine group, coordination to platinum was effected by activation of [Pt(en)Cl_2] with $AgNO_3$ in DMF followed by reaction with pyridine **L1** for 24 h at room temperature. Purification of the crude product was performed by preparative HPLC to afford complex **1** in >95% purity (λ 472 nm). In contrast to pyridine **L1**, complex **1** is readily soluble in water and could be stored as a 5 mM solution in water (containing 20 mM NaCl) with no decomposition observed by HPLC after storage for at least two months at 4 °C.

Conjugation of complex 1 to trastuzumab

The reactivity of complex **1** toward trastuzumab was first assessed in tricine/$NaNO_3$ buffer, typically employed for conjugation of platinum(II) complexes to albumin and lysozyme.[4a,g–j] When trastuzumab, used in its commercial formulation, was incubated with complex **1** (20 equiv) for 2 h at 37 °C, conjugation was clearly observed by size-exclusion chromatography (SEC), and no aggregation was observed. After purification by PD-10 column, an NBD/mAb ratio of 2.8 was found, as derived from the absorption of the NBD group (λ 472 nm) and the protein (λ 280 nm, corrected for absorption of complex **1** at this wavelength). Alternatively, the NBD/mAb ratio was determined by exploiting the hydrolytic instability of the NBD fluorophore at high pH.[11] Briefly, overnight incubation of the purified conjugate at pH 10.5 resulted in complete hydrolysis of the NBD label to NBD-OH (Figure 3). Separation by SEC then allowed

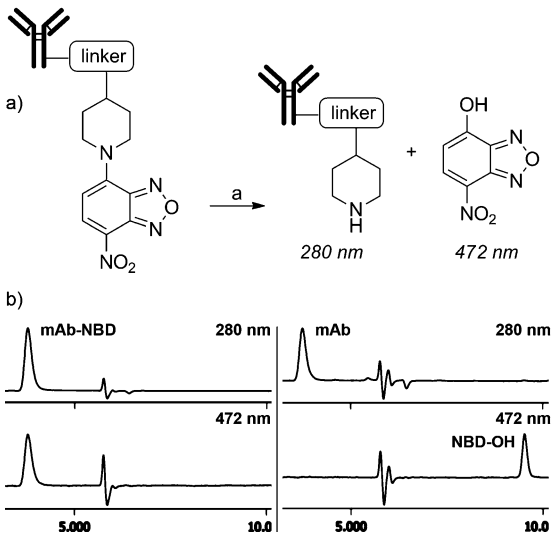


Figure 3. Method to assess NDB/mAb ratio by UV measurement of NBD-OH (λ = 472 nm) and mAb (λ = 280 nm). a) Hydrolysis of **1**–trastuzumab. *Reagents and conditions*: a) glycine buffer (pH 10.5), 37 °C, 16 h. b) SEC chromatograms of the hydrolysis of **1**–trastuzumab (mAb–NBD) at t = 0 (left) and t = 16 h (right) at pH 10.5.

determination of the relative concentration of protein and NBD-OH. Using this method, an identical NBD/mAb ratio of 2.8 was found. The latter method was preferred and used for subsequent analyses.

Despite the practical advantages of using the NBD group, its hydrolytically labile nature also imposes some limitations. Control experiments showed 8 and ~0.2% hydrolysis of the NBD

group at pH 8.0 and 7.1, respectively, after incubation at 37 °C for 2 h, indicating that NBD/mAb ratios of conjugations performed at pH 8.0 are a slight underestimation (data not shown).

Effect of buffer and pH on conjugation efficiency

To explore the effect of pH and buffer on the reaction of complex **1** with trastuzumab, reactions were performed in three different buffers: MES, HEPES, and tricine, at pH 5.8–8.0 with overlapping pH ranges to discriminate between buffer and pH effects. As shown in Table 1, a clear pH effect was observed,

Table 1. Conjugation of complex **1** to trastuzumab: effect of buffer and pH on conjugation efficiency.[a]

Buffer	pH[c]		NBD/mAb
	Buffer	Observed	
MES	5.5	5.8	1.3
MES	6.4	ND	1.8
HEPES	6.5	6.3	1.7
HEPES	7.5	7.1	2.5
HEPES	8.5	ND	2.8
tricine	8.5	8.0	2.8
tricine + thiourea[b]	8.5	ND	0.1

[a] *Reagents and conditions*: trastuzumab 83.1 μM, complex **1** (20 equiv), buffer (8 mM), NaCl (6.5 mM), 37 °C, 2 h. [b] Conjugation in the presence of thiourea (10 mM). [c] pH measured at the start of reaction at 20 °C.

with higher NBD/mAb ratios observed at higher pH. Because coordination of platinum(II) to sulfur donors such as methionine and disulfides is believed to be relatively insensitive to pH, this trend implies that histidine groups, with pK_a values typically in the range of 5.5–7.5,[12] are likely involved in binding to complex **1**.

MES and HEPES buffers reportedly show negligible metal binding, whereas tricine does show some affinity for metals, such as Cu^{2+}.[13] Comparison of overlapping pH ranges for these three buffers, however, showed no significant difference in the number of NBD groups introduced. To exclude binding of complex **1** to trastuzumab via pathways not mediated by platinum(II), the reaction was also performed in the presence of thiourea, an excellent ligand for platinum(II). These conditions effectively inhibited the conjugation reaction for complex **1**, indicating that binding is indeed platinum mediated. Reaction conditions with pH > 8.0 were not explored due to the unstable nature of the NBD group under basic conditions.

Effect of salt on conjugation efficiency

Both organic and inorganic salts are routinely used in buffers and as stabilizers in antibody formulation. Depending on salt type and concentration, their presence could significantly impact the efficiency of the conjugation reaction. In previous studies,[4a,g–j] $NaNO_3$ was often employed in conjugation reactions of platinum-based linkers with lysozyme or albumin. However, to the best of our knowledge, a study on the effect of salt type on the reaction of monovalent platinum(II) complexes with proteins has never been reported.

To investigate this in greater detail, complex **1** in water (Milli-Q) was reacted with trastuzumab in tricine buffer in the presence of a variety of salts (Table 2). Notably, trastuzumab is

Table 2. Conjugation of complex **1** to trastuzumab: effect of added salt on conjugation efficiency.[a]

Salt	Conc. [mM]	NBD/mAb[b]
–	–	2.7
Na_2SO_4	30	5.1
NaOAc	30	3.1
NaCl	6.5	2.8
NaCl	30	3.2
$NaNO_3$	30	3.6 (3.5/3.7)[b]
$NaClO_4$	30	4.1 (3.6/2.6)[b]
$NaCl/NaNO_3$	6.5/30	3.1
$NaCl/NaClO_4$	6.5/30	3.7
$NaCl/Na_2SO_4$	6.5/30	4.1

[a] *Reagents and conditions*: trastuzumab 83.1 μM, complex **1** (20 equiv), tricine (8 mM, pH 8.5), 37 °C, 2 h. [b] Salt concentration: 60/120 mM.

formulated in a histidine buffer which typically results in a minimum chloride concentration of ~0.9 mM for the conjugation reactions listed in Table 2. Interestingly, the salt type proved to have a pronounced effect on the NBD/mAb ratio obtained. Whereas addition of NaCl and NaOAc only showed a modest increase at 30 mM, $NaNO_3$, $NaClO_4$, and especially Na_2SO_4 showed a substantially higher NBD/mAb ratio. Increasing the salt concentration to 60 and 120 mM was ineffective for $NaNO_3$, whereas a strong decrease was observed for $NaClO_4$ at 120 mM. The effect of NO_3^-, ClO_4^-, and SO_4^{2-} was somewhat diminished when the Cl^- concentration was increased to 6.5 mM.

The significant differences between the various salts are not easily explained if only the interaction of the salt with the platinum complex is considered. Apparently, at the concentrations used, none of the salts tested interfere with the binding of complex **1** to trastuzumab, as the reaction is least efficient when no salt is added. One could speculate that certain salts could protect the platinum complex from aquation, which at pH 8.0 is expected to result in deprotonation of the water ligand ([Pt(dien)(H_2O)]; $pK_a = 6.0$)[19c] to give an unreactive hydroxide complex. It is, however, well known that in contrast to NO_3^- and SO_4^{2-}, ClO_4^- does not coordinate to platinum(II).[14]

An intriguing and more likely alternative to explain the difference in conjugation efficiency is the interaction of the salt with the protein. Recently, Weis and co-workers[15] reported on the effect of Hofmeister series salts on the local flexibility of an IgG1 monoclonal antibody, as measured by hydrogen/deuterium exchange coupled to mass spectrometry. Compared with Cl^- (0.1 M), both SO_4^{2-} and SCN^- were found to increase the flexibility of a model IgG1 at 0.5 M, albeit not necessarily in the same areas. At 0.5 M, Cl^- was found to induce an overall decrease in flexibility of IgG1. Zhang-van Enk et al. recently

showed that, depending on pH, SO_4^{2-} and SCN^- strongly destabilize the human IgG1 Fc fragment at concentrations below 50 mM.[16] When the results from Table 2 are aligned with the Hofmeister series of anions, one could speculate that the higher conjugation efficiencies for SO_4^{2-} and ClO_4^- might, at least in part, be explained by an increase in the flexibility of IgG1, thereby facilitating access to coordination sites in the protein for platinum. (Figure 4).

Hofmeister series of anions									
F^-, SO_4^{2-}	>>	$CH_3CO_2^-$	>	Cl^-	>	NO_3^-	>>	ClO_4^-	>	SCN^-
5.1		3.1		3.2		3.6		4.1		
observed NBD/mAb ratio										

Figure 4. The Hofmeister series of anions and observed NBD/mAb ratio at pH 8.0 as shown in Table 2.

Stability and mass spectrometry analysis of 1–trastuzumab

Among the various potential binding partners in proteins, histidine, methionine, and cysteine are expected to be preferential binding sites for platinum(II) at near neutral pH.[6] The stability of the respective platinum–amino acid complexes does not necessarily have to be the same. For instance, it is believed that in cisplatin therapy, the rescue agent sodium diethyldithiocarbamate (DDTC) is capable of reversing the binding of platinum(II) to methionine residues, but not for platinum(II)–cysteine bonds.[4,6e,17] This raises the question if the conjugates prepared display a heterogeneous stability, with both weakly and strongly bound Pt complexes present in the conjugate. Indeed, when **1**–trastuzumab was stored in phosphate-buffered saline (PBS) for 48 h at room temperature, analysis by SEC showed a partial release of the NBD linker, as shown in Figure 5. It was hypothesized that a post-treatment step with an S-donor ligand might be able to remove weakly bound platinum complexes causative to this release.

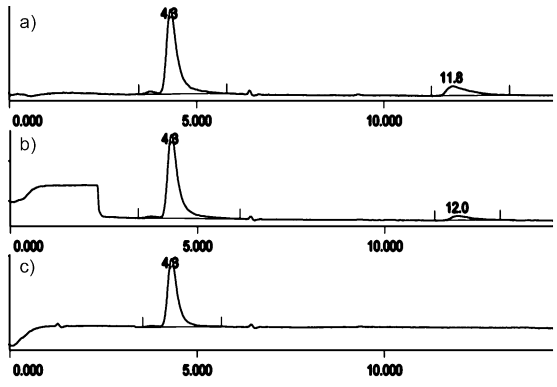


Figure 5. SEC chromatograms of **1**–trastuzumab (NBD/mAb: 3.2) after PD-10 purification and storage in PBS at RT for 48 h. Reference retention times **1**–trastuzumab: 4.3 min, NBD-OH: 9.4 min, complex **1**: 10.6 min, pyridine **L1**: 16.2 min (not detected). a) No post-treatment step before PD-10 purification. b) Post-treatment step with methionine (50 mM) for 60 min at 37 °C before PD-10 purification. c) Post-treatment step with thiourea (10 mM) for 30 min at 37 °C before PD-10 purification.

To this end, **1**–trastuzumab was prepared under identical conditions, and the crude reaction mixture was treated with methionine or thiourea for 30–60 min before being purified by PD-10 column. Whereas methionine (50 mM) only partially decreased the release of NBD label for **1**–trastuzumab upon storage in PBS, thiourea (10 mM) completely abrogated release of the NBD label. These data show that, relative to methionine, thiourea more efficiently removes weakly bound complex **1**. As is evident from the lower NBD/mAb ratios listed in Table 3, this procedure partially reverses the binding of complex **1** to trastuzumab. Because extending the thiourea treatment from 30 to 60 min at a fivefold higher concentration did not result in a further decrease in the NBD/mAb ratio, the thiourea apparently only selectively removes a weakly bound subpopulation of complex **1** (Table 3).

Table 3. Incubation of **1**–trastuzumab with S-donor ligands to remove weakly bound platinum complexes.[a]

Competitor	Conc. [mM]	Incubation time [min]	NBD/mAb
–	–	–	3.2
methionine	50	60	2.7
thiourea	10	30	2.0 (2.5)[b]
thiourea	50	60	2.0

[a] *Reagents and conditions*: trastuzumab 83.1 μM, complex **1** (20 equiv), tricine (8 mM, pH 8.5), 37 °C, 2 h, then addition of competitor S-donor compound and incubation for time specified. [b] Conjugation performed with $NaClO_4$ (30 mM).

Besides weakly bound complex **1**, nonspecific binding of unreacted complex **1** to trastuzumab could be an alternative explanation for these findings. In this scenario, thiourea and methionine would rapidly scavenge the remaining complex **1**, resulting in a more hydrophilic dicationic complex with possibly decreased affinity for the lipophilic protein surface. Although difficult to disprove, complex **5** was synthesized to assess the propensity of NBD–platinum complexes for nonspecific binding to trastuzumab (Figure 6). Reaction of the more lipophilic complex **5** with trastuzumab afforded an NBD/mAb ratio of <0.1. Because only an insignificant amount of complex **5** was still present after PD-10 purification, it is unlikely that the thio-

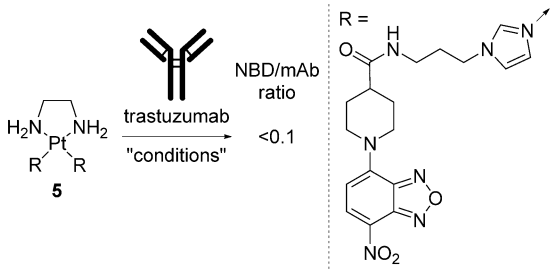


Figure 6. Lipophilic complex **5** used in control experiment to exclude nonspecific (not platinum-mediated) binding to mAb. *Reagents and conditions*: trastuzumab (83.1 μM), complex **5** (20 equiv), tricine (8 mM, pH 8.5), 37 °C, 2 h.

urea step removes a significant amount of nonspecifically bound intact complex **1**. Altogether, these results indicate that thiourea effectively removes weakly bound complex **1** from the antibody.

The binding of complex **1** to trastuzumab was further characterized by mass spectrometry analysis. Figure 7 shows the ESI-TOF-MS spectrum of thiourea-treated, deglycosylated **1**–trastuzumab after hydrolytic cleavage of the NBD group in glycine buffer, pH 10. The average mass differences between adjacent peaks is 473.7 Da, which corresponds well with the 474 Da expected for the residual linker. The NBD/mAb ratio of 1.6 is in good agreement with UV/Vis analysis (1.7 NBD/mAb).

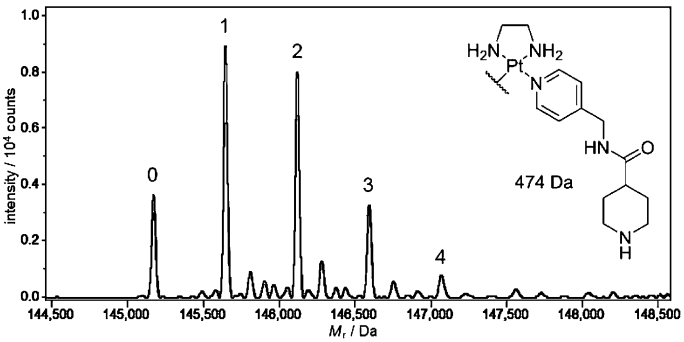


Figure 7. ESI-TOF-MS spectrum of thiourea-treated (10 mM, 30 min), deglycosylated **1**–trastuzumab after hydrolytic cleavage of the NBD label.

Effect of excipients in mAb formulation on conjugation efficiency

The trastuzumab used in this study was reacted without prior removal of excipients. One of these excipients is histidine, which in theory, could react with platinum and decrease the efficiency of the conjugation reaction. To investigate this, trastuzumab was reacted with complex **1** in the presence of a tenfold higher concentration of histidine with respect to the amount that is normally introduced to the reaction mixture by the trastuzumab formulation. As shown in Table 4, the approximate tenfold excess of histidine with respect to platinum led to a decrease in conjugation efficiency relative to the conjugation in which excipients were removed by spin filtration. Post-conjugation treatment with thiourea prior to PD-10 purification, however, resulted in nearly identical NBD/mAb ratios for both reactions. These results indicate that histidine either acts as a scavenger for weakly bound platinum, like thiourea, or that it competes with a subpopulation of protein coordination sites for binding to platinum. Because mAbs are frequently formulated in a phosphate buffer, it was also investigated if the conjugation reaction with trastuzumab can be performed in PBS at pH 7.5. Interestingly, despite the relatively high Cl^- concentration, PBS only marginally decreased the conjugation efficiency and presents a viable alternative to tricine or HEPES buffers.

Table 4. Excipient effects on the conjugation of complex **1** to trastuzumab.[a]

Buffer	pH	Histidine [mM]	NBD/mAb[c]
tricine	8.0	16	2.5 (1.9)
tricine[b]	8.0	–	3.8 (2.0)
PBS	7.5	–	3.5 (1.8)
PBS[b]	7.5	–	3.4 (1.8)

[a] *Reagents and conditions*: trastuzumab (83.1 μM), complex **1** (20 equiv), buffer/added salt: tricine (8 mM, pH 8.5)/NaCl (6.5 mM), or 1× PBS/NaCl (6.5 mM), 37 °C, 2 h. [b] Excipients removed by spin filtration. [c] Values in parentheses: NBD/mAb ratio after post-conjugation treatment with thiourea (10 mM) for 30 min.

Synthesis of a diverse set of platinum complexes

To investigate the effect of the type of coordinating group on the efficiency of the conjugation reaction, a diverse set of NBD ligands, covering *N*-heteroaromatic, *N*-aliphatic amine, and thioether coordinating groups, were synthesized and complexed with $[Pt(en)Cl_2]$ (Figure 8). Whereas synthesis of the heteroaromatic complexes **6**, **7**, and *N*-alkylamine complex **8** was straightforward, dipeptide ligand **L5** afforded a more complex reaction mixture, most likely due to the formation of diastereomers. According to HPLC analysis, two products formed initially at a 1:1 ratio, which after 48 h at 60 °C had shifted to a ratio of 2:5. The minor product appeared to be in rapid equilibrium with its more polar aquated form, which, after incubation of the crude reaction mixture with Milli-Q water, allowed isolation of the major product by preparative HPLC in 44% yield. Spectral data were in full agreement with structure **9**, but we were unable to unambiguously determine the relative stereochemistry. Once in solution, the purified product slowly equilibrated

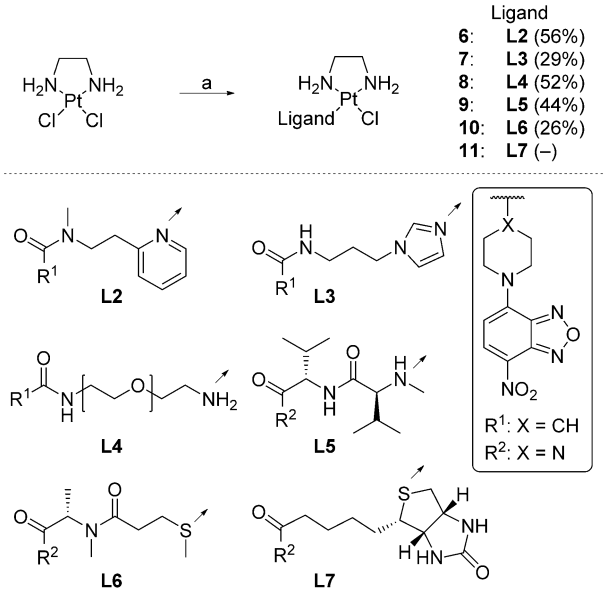


Figure 8. Synthesis of complexes **6**–**11**. *Reagents and conditions*: a) $AgNO_3$, DMF, RT, 16 h, then ligands **L2**–**L7**, RT–60 °C, 24–48 h.

again to give the same product distribution as before purification. Thioether complex **10** was also successfully synthesized, and, despite the strong destabilizing effect of thioethers on platinum,[18] was found to be stable in 20 mM NaCl at 4 °C for >11 months as determined by HPLC. In this respect, the instability of the product formed upon reaction of the bulkier, biotin-derived thioether **L7** with $[Pt(en)Cl_2]$ was rather unexpected. After preparative HPLC, the product was obtained in ~80% purity (HPLC) which largely reverted back to starting material upon storage in 20 mM NaCl for 48 h at room temperature. Because of its unstable nature, no conjugation experiments were performed with complex **11**.

Effect of coordinating group on conjugation efficiency

As reported earlier, the coordination of 2-substituted pyridines to platinum(II) is believed to greatly decrease the reactivity of platinum(II) by steric shielding at the axial site, especially toward soft nucleophiles such as thiols.[19] Indeed, as shown in Table 5, complex **6** proved virtually unreactive toward trastuzumab, illustrating that this substitution pattern also greatly decreases the reactivity of platinum(II) toward proteins. Furthermore, when compared with complex **1**, the poor NBD/mAb ratio obtained for complex **6** also reinforces the notion that nonspecific binding is unlikely to occur with these complexes. The *N*-alkylated imidazole complex **7** represents another example of a heteroaromatic system that can be successfully conjugated to trastuzumab with an efficiency similar to that of complex **1**.

Table 5. NBD/mAb ratios for complexes **6**–**10** with trastuzumab.[a]

Complex:	**6**	**7**	**8**	**9**	**10**
NBD/mAb:[b]	0.2 (0.2)	3.1 (1.9)	1.9 (1.5)	3.2 (2.9/2.9[c])	2.1 (1.1/0.1[c])

[a] *Reagents and conditions*: trastuzumab 83.1 μM, complex **6**–**10** (20 equiv), tricine (8 mM, pH 8.5), 37 °C, 2 h. [b] Values in parentheses: NBD/mAb ratio after post-conjugation treatment with thiourea (10 mM) for 30 min. [c] Thiourea (50 mM), 60 min.

Somewhat surprisingly, the sterically less encumbered primary amine **8** reacted less efficiently. According to HPLC analysis, complex **8** is more susceptible to aquation than complexes **6** and **7**, which at the reaction pH is likely to result in faster deactivation of the complex via aquation followed by deprotonation. The sterically more demanding secondary amine **9** showed a remarkable improvement in conjugation efficiency, which, in contrast to the other complexes explored, only marginally decreased upon challenging the conjugate with thiourea. Notably, no decrease in NBD/mAb ratio was observed upon extended treatment time and higher thiourea concentration.

An interesting observation was made for thioether complex **10**. Thiourea treatment of **10**–trastuzumab resulted in a rather large decrease in NBD/mAb ratio which raised the question if the complex was inherently unstable. Indeed, a fivefold higher thiourea concentration and 60 min incubation time resulted in nearly complete removal of the NBD label from trastuzumab. When thiourea treated **10**–trastuzumab, with <0.1 NBD/mAb, was subjected to ICP-MS analysis, a platinum/mAb ratio of 2.5 was observed. This result clearly shows that the platinum–thioether bond is readily cleaved in **10**–trastuzumab upon thiourea treatment. This, together with the unstable nature of the biotin-derived thioether complex **11**, make it seem unlikely that thioether-coordinated platinum(II) complexes allow stable coordination to proteins. The difference in stability for pyridine complex **1** and thioether complex **10** is most readily explained by the stronger destabilizing trans-effect of thioethers relative to pyridines.[18]

Effect of platinum coordination on antigen binding properties of trastuzumab

Finally, to determine if coordination of NBD–platinum(II) complexes to trastuzumab decreases its affinity for its cognate antigen Her2, the dissociation constant for trastuzumab and **1**–trastuzumab (3.3 NBD/mAb) was determined by surface plasmon resonance (SPR) array imaging. The equilibrium dissociation constant found for trastuzumab ($K_D = 35 \pm 5$ pM) and **1**–trastuzumab ($K_D = 38 \pm 8$ pM) was very similar and shows that coordination of complex **1** to trastuzumab does not interfere with antigen binding.

Conclusions

The synthesis of a diverse set of $[Pt(en)(L\text{-NBD})Cl](NO_3)$ complexes is reported, and the conjugation of these complexes to the model IgG1 trastuzumab investigated. It was found that the coordinating group used for coordinating the NBD label to $[Pt(en)Cl_2]$ strongly affected the efficiency of the conjugation reaction with trastuzumab, as well as the stability of the resulting conjugate. *N*-Heteroaromatic and aliphatic amine derived complexes could be successfully conjugated to trastuzumab, with the exception of a 2-substituted pyridine, which proved unreactive. The latter result reinforces the notion that this substitution pattern strongly decreases the reactivity of platinum(II) toward proteins. Of the various N-coordinated complexes tested, the primary amine afforded the lowest NBD/mAb ratios, whereas a more hindered secondary amine proved most efficient. In contrast to N-coordinated complexes, S-coordinated thioether complexes were found to be kinetically labile and did not afford conjugates stable toward thiourea treatment. These results imply that coordination to sulfur in methionine residues and disulfide bridges is probably best avoided if a highly stable conjugate is required. In this respect, the partial release of the NBD label after conjugation is noteworthy and is hypothesized to mainly originate from kinetically labile platinum–methionine and platinum–disulfide complexes. Inclusion of a post-treatment step with thiourea could effectively remove labile platinum(II) complexes, and resulted in conjugates that are stable in PBS for at least 48 h at room temperature.

The effect of reaction conditions on the efficiency of the conjugation reaction was also explored. A wide range of buffers was tolerated, and no correlation between buffer type and conjugation efficiency was observed for MES, HEPES, and tricine. However, the pH strongly affected the conjugation efficiency, and pH 8.0 afforded the highest NBD/mAb ratios in the absence of added salt. The efficiency of the reaction, however, was improved by the presence of certain salts in the reaction. Of the various salts tested, NO_3^-, ClO_4^-, and especially SO_4^{2-} improved the efficiency of the reaction at 30 mM concentration. This effect is postulated to arise from an increased flexibility of peptide chains in the presence of these salts. Finally, SPR measurements showed that under the conditions used, binding of complex **1** does not compromise the immunoreactivity of trastuzumab, illustrating that further investigations on the use of $[Pt(en)Cl_2]$ as linker in the field of antibody–drug conjugates is warranted. Likewise, to reveal the full potential of this concept, the identification of binding sites for platinum(II) in monoclonal antibodies is of great interest and is currently being pursued.

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