A Ligand-Directed Nitrophenol Carbonate for Transient In Situ Bioconjugation and Drug Delivery

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Abstract: Here we report the first use of ligand-directed proximity accelerated bioconjugation chemistry in the tandem delivery and release of a therapeutic payload. To do this we designed a nitrophenol carbonate for ligand-directed in situ bioconjugation of a prodrug payload to a protein. The transient nature of our conjugation chemistry renders the protein a depot for time-dependent release of active drug following hydrolysis and self-immolation. In our model system, using an immunostimulant prodrug, biotin ligand, and avidin protein, we observe time-dependent release of bioavailable immunostimulant both spectroscopically and with an immune cell line over 48 h. Avidin co-crystallized with the biotin directing group verified the binding pose of the ligand and offered insight into the mechanism of in situ bioconjugation. Overall, this scaffold warrants further investigation for the time-dependent delivery of therapeutics and use in protein ligand pairs beyond biotin and avidin used for this work.

Bioconjugates are an alluring therapeutic modality that, relative to small molecules, suffer drawbacks in production including high cost, poor batch reproducibility, and poor shelf life; all of which hinder the development of bioconjugates as drugs. Despite these drawbacks, the benefits of tissue specificity, synergistic effects of the biomolecule and drug, and increased biological half-life have still pushed researchers to explore bioconjugate drug delivery. More recently, in situ bioconjugates have been explored using ligand-directed small-molecule probes whereby ligand-protein complexation leads to covalent affinity labeling resulting in a bioconjugate. We envisioned that if this concept could be expanded to develop an affinity labeling platform that yields in situ bioconjugates capable of time-dependent drug release, this could bypass the drawbacks of producing bioconjugate drugs while still harnessing the therapeutic benefits.

Ligand-directed affinity labeling techniques have been pioneered by Hamachi and coworkers. They have developed multiple electrophilic moieties over the past

Figure 1. Drug delivery via ligand-directed nitrophenol carbonate (LDNPC) chemistry involves 3 steps to directed release of active payload. Step 1. Addition of LNPC reagent to target protein results in protein-ligand complex. Step 2. Nucleophile (Nu) peripheral to the binding site attacks LNPC reagent displacing the nitrophenolate-modified ligand, resulting in a covalently modified target protein. The nitrophenolate-modified ligand is free to dissociate. Step 3. Hydrolysis and self-immolation result in release of bioavailable payload at the location of the covalently modified protein. In this work X is an alanine NH.
decade capable of labeling nucleophilic amino acid side chains on proteins with small-molecule probes peripheral to the ligand binding site.\[6-16\] It is hypothesized that upon ligand binding, an electrophilic moiety on the ligand-directed reagent is attacked by a nucleophilic side chain and a leaving group displaced, leading to the covalent attachment of a probe to the target protein. They have demonstrated success with soluble proteins such as carbonic anhydrase as well as membrane bound proteins such as the folate receptor with examples of in situ, in vitro, ex vivo, and in vivo labeling.\[11-17\] With these ligand-directed probes in mind, we designed a scaffold (Figure 1) that allows for time-dependent drug delivery via a transient in situ bioconjugate formed by ligand-directed affinity labeling.

Ligand-directed nitrophenol carbonate (LDNPC) chemistry was built around the leaving group ability of nitrophenol, formaldehyde, cyanide, and ethylene glycol. LDNPC reagents exhibit excellent bench stability as a lyophilized powder and modest solution stability. Thus, LDNPC reagents mitigate the drawbacks of conventional bioconjugate drug production while retaining their alluring benefits via in situ synthesis.

For this proof-of-concept work, the protein ligand pair of avidin-biotin was chosen because: 1) the avidin-biotin crystal structure is known, and verified the presence of possible peripheral nucleophiles (Nu) to the binding site.\[19\] 2) The high affinity of avidin for biotin (Kd ≈ 10^{-15} M)\[20\] was hypothesized to lead to rapid complexation and therefore rapid covalent labeling. 3) Due to the multivalency of avidin-biotin, we believe that LDNPC chemistry could add a new tool to the many established applications that utilize avidin-biotin biotechnology.

The imidazoquinoline immunostimulant Imiquimod (IMQ)\[21-24\] was chosen as payload for our proof-of-concept LDNPC reagent because our previous work with enzyme-directed imidazooquinolines allowed us to estimate imidazoquinoline behavior as prodrugs.\[25,26\] Due to the well-defined structure activity relationship of the imidazoquinoline drug class,\[27,28\] we hypothesized that linkage at the C4 aniline would lead to abrogated immunostimulatory activity and therefore allow the LDNPC reagent, as well as the covalently labeled avidin complex to serve as a prodrug of IMQ. Furthermore, the IMQ payload is an active immunostimulant for the RAW-Blue murine macrophage cell line (RB Cells) which links...

Figure 2. a) Synthetic route to ligand-directed nitrophenol carbonate (LDNPC) (8). Fragment A i: EDC-HCl, NHS, DMF, RT 24 h. Fragment B ii: Methylal, HCl (g), HCl conc., H2SO4 conc., 72 °C, 4 h. iii: acetonitrile, 5 M KCN in H2O, 0 °C 30 min, 60 °C 30 min. iv: 1) BH3-THF 1 M in THF, 100 °C, 4 h. 2) HCl 0.8 M in MeOH, 100 °C, 12 h. Fragment C v: p-nitrophenyl chloroformate, pyridine, DCM, 90 °C, 24 h. vi: Imiquimod, THF, MW Irradiation: 90 °C, 55 min, 1 bar. (A+B) vii: DIPEA, DMF, RT, 18 h. (A+B) + C viii: DIPEA, DMF, RT, 24 h b) Degradation kinetics of 120 µM LDPN (8) was measured as release of nitrophenolate (7) in the presence of Avidin (Red, 0.42 mg/mL), Avidin + Biotin (Blue, 0.42 mg/mL, 12 uM), Bovine Serum Albumin (BSA) (Black, 0.42 mg/mL) or citrate phosphate buffer (Purple, 50 mM pH 7). The effects of avidin on LDPN (8) are statistically significant compared to BSA and Buffer (p < 0.05 calculated at 2, 5, 10, 15, 20 min; error bars are standard deviation of the mean for triplicate experiments). See supporting information for longer timepoints.
activation of the transcription factor NF-κB to secreted embryonic alkaline phosphatase which can be quantified with colorimetric phosphate substrates. For these reasons, the utility of LDNPC chemistry as a drug delivery technique can be proven utilizing a biotin directing group to render avidin as a time-dependent imidazoquinoline prodrug in situ.

The biotin-LDNPC-IMQ reagent (8) is synthesized via the following convergent synthetic approach (Figure 2a). In which ligand (fragment A) can be combined with nitrophenol moiety (fragment B) and the resulting biotinphenol (fragment A⋅B) can act as Nu to attach the IMQ payload (fragment C) to yield LDNPC (8). Fragment A is prepared in one step by the known procedure of activating the carboxylate of biotin as succinimidyl ester (1) with EDC in DMF.[29] Fragment B is prepared over three steps from p-nitrophenol (PNP). Chloromethylation of PNP is accomplished under acidic conditions with methylyal and HCl gas.[30] The resultent benzyl chloride (2) is displaced by cyanide to yield benzyl cyanide (3) which is subsequently reduced with borane to yield primary amine (4).[31] Fragment C is synthesized over two steps from ethylene glycol and p-nitrophenyl chloroformate, yielding the ethylene glycol dicarbonate (5). Symmetry of (5) is broken under microwave conditions with Imiquimod to yield (6) the intermediate self-immolative spacer (fragment C). Fragments A and B are combined in DMF with tertiary amine base to yield biotin-phenol (7), and then reacted with fragment C under basic conditions, and after 24 h equilibrium is reached and LDNPC (8) is obtained in 8.7% yield over 5 synthetic steps from PNP. Complete synthetic procedures and characterization may be found in the supporting information.

With LDNPC (8) in hand, we next characterized the stability in aqueous medium. First, the pKa of biotinphenol leaving group (7) was determined to be 8.02 by titration with sodium hydroxide in water (Figure S1) indicating that LDNPC (8) should resist base mediated hydrolysis below this pH value. Aqueous stability of (8) was monitored over 48 h at 37 °C in citrate phosphate buffer over a range of pH: 4, 5, 6, and 7, typical of cellular organelles,[32] and release of IMQ from direct hydrolysis was quantified by HPLC. LDNPC (8) displayed a trend of increasing aqueous stability with decreasing pH and, at pH 7, released 0, 5, and 77% of total IMQ from direct hydrolysis at 4, 12, and 48 h respectively (Figure S2). The stability of the complex was adequate over 12 h considering the attenuated yet still high affinity of our LDNPC reagent for avidin. This is further supported by Hamachi et al. who show saturated covalent labelling with their ligand-directed acyl imidazole chemistry within 7 h for carbonic anhydrase I.[17]

Figure 3. X-ray crystal structure of Biotin-Phenol (7, grey) bound to avidin (1.58 Å resolution). a) Flexible loop regions around nitrophenolate moiety, proximal nucleophiles (Nu) (within 12 Å of phenolate) are shaded in purple and include residues S41,102, T40,113, and K111. Only Nu from 1 monomer are pointed out. Monomer A and C are shaded lavender and light green, respectively. b) Highlight of residues A39, S101, R114 (lime green) directly implicated in stabilizing nitrophenolate in the avidin binding pocket. c) View showing electrostatic surface. Distance between ligand bound to monomer A and C is only 8.4 Å between the aryl rings. This view also shows how the nitrophenolate moiety is positioned just outside the Biotin pocket in a solvent accessible region.
We next examined the influence of avidin on the release of IMQ from degradation of LDNPC (8). Due to the 4-nitrophenoletic leaving group of (7), it was observed that cleavage of the carbonate phenol bond by Nu liberates a colorimetric indicator of degradation. We exploited this property to follow degradation of (8) in pH 7 buffer, with avidin or bovine serum albumin (negative control). In the presence of avidin, significant (80 µM) release of (7) was measured compared to minimal hydrolytic degradation with BSA or buffer alone (< 20 µM), emphasising nuclophilic attack following ligand complexation. Expectedly, competition from biotin attenuates release (Figure 2B, S3). This difference was attributed to the enzyme-like activity of the affinity labelling where ligand binding is associated to a mechanistic cue that induces a covalent change to the protein. After using colorimetry to observe the initial formation of bioconjugate over 90 min, we next used HPLC to quantify the release of IMQ over 48 h with and without avidin. Both solutions showed measurable amounts of IMQ release. Interestingly, the sample with avidin showed a significant reduction in IMQ released (Figure S4), and these measurements combined with the colorimetric differences lead us to conclude that avidin is successfully labelled by LDNPC (8). Based on the possible nucleophilic side chain addition to (8), we would expect the resulting bioconjugate to be considerably more stable than the carbonic acid formed from direct hydrolysis of (8). Therefore, we reason that the results of this experiment are consistent with transient covalent attachment of the self-immolative spacer and IMQ to avidin.

After confirming that our LDNPC reagent could label avidin in situ, we turned to structural biology to gain insight on the possible residues acting as Nu. Due to hydrolysis of the bioconjugate over the crystallization period we were unable to obtain co-crystals of avidin with (8). We also attempted to crystalize avidin and soak in LDNPC (8). Unfortunately, X-ray diffraction of these crystals did not provide complete electron density maps. In lieu of this, we proceeded to co-crystallize avidin with the biotin-phenol leaving group (7). This approach resulted in a 1.58 Å resolution crystal structure of the avidin-biotin-phenol (7) complex (PDB: 6XND, Figure 3, Table S1).

As expected, the biotin moiety of (7) is situated in a similar binding pose as biotin,[19] buried within the binding pocket of the eight-stranded anti-parallel β-barrel. Emerging from the pocket and surrounded by solvent accessible flexible loop regions is the nitrophenol moiety (Figure 3A). Isothermal titration calorimetry shows the dissociation constant of the ligand to be $K_d = 2.25 \times 10^{-7}$ M (Figure S6), considerably weaker than biotin.

The nitrophenolate appears to be stabilized by hydrogen bonds from S101 and R114 to the phenol, as well as a Van der Waals interaction between A39 and the nitro group (Figure 3B). Possible Nu in the region (within 12 Å of phenolate) include S40, S41, S101, S102, and K111. All are constituents of dynamic loop regions and therefore all potentially capable of being labeled by LDNPC (8). Interestingly, the interface between the monomers of avidin position two equivalents of ligand within the same solvent accessible pocket, with the aromatic rings separated by only 8.4 Å in the static crystal structure (Figure 3C). This overcrowded region could also drive hydrolysis to release the payload thereby relieving this steric strain and returning the protein to its native state. Taken together, this data concretely proves that the modified biotin reagent remains a ligand for avidin and that proximal Nu to the carbonate moiety of LDNPC (8) are abundant.

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**Figure 4.** a) Workflow of time-dependent immunogenicity assay. Avidin samples are loaded with (8) and incubated for 60 s, at 37 °C, protein then separated with 10 kDa MWCO centrifuge device: 14,000 RCF, 5 min, 37 °C. Protein reconstituted in 300 µL PBS and then again separated with centrifuge device, (same conditions) and wash repeated three times. The samples are then incubated for indicated time prior to running a RB Cell assay to measure immunogenicity. b) Results of assay show time-dependent immunogenicity from avidin loaded with (8) (Gray). Theoretical loading was 50 µM of IMQ (Red), comparable immunogenicity is observed from bioconjugate in 12-24 h of incubation. Avidin has small, but significant, immunogenicity (p<0.001) on its own compared to blank.
We next sought to demonstrate the prodrug nature of avidin labeled with LDNPC (8), and time-dependent release of IMQ from the resultant in situ bioconjugate. First, to show the abrogated immunogenicity from LDNPC (8) in direct comparison to IMQ we treated RB cells with equimolar concentrations (1 – 100 µM) of each respective compound (Figure S7). The RB cell assay is conducted over 16 h of incubation time, and so we expected some non-specific hydrolysis in the cell media. The results show abrogated activity of LDNPC reagent compared to IMQ, with significantly reduced immunogenicity for the LDNPC reagent at 50 and 100 µM (p <0.001). Even at lower concentrations (1, 5, and 10 µM) the trend of reduced immunogenicity is conserved, albeit at lower significance thresholds. With proof of abrogated activity, we next designed an experiment to capture the time-dependent release of IMQ in IMQ standards. With proof of abrogated activity, we next designed an experiment to capture the time-dependent release of IMQ and therefore time-dependent immunogenicity. In the same assay, we wanted to highlight that in situ bioconjugation results in biomolecule directed immunogenicity. To accomplish these aims, we utilized a 10 kDa molecular weight cut off centrifugal filter device (CFD). The workflow of the assay (Figure 4a), begins by incubating avidin with LDNPC (8) for 60 s at 37 °C. After sufficient time for complexation, the loading filtrate was spun through the CFD effectively isolating the IMQ-avidin bioconjugate. The bioconjugate was then washed, concentrated, reconstituted in buffer, and incubated for the indicated time (72 – 0 h, 12 h increments) before being used in a RB cell assay that was developed for 16 h. By use of the CFD we have shown that the results of the assay are caused by bioconjugation of the LDNPC reagent and avidin. Any uncomplexed LDNPC reagent, and likewise any IMQ released by non-specific hydrolysis (both < 1 kDa) is washed through the membrane. Therefore, we can say that any observed immunogenicity in the assay is resultant from in situ bioconjugation and subsequent hydrolysis from avidin. We anticipated that longer incubation times of the bioconjugate in buffer would result in greater payload release and therefore higher immunogenicity approaching the theoretical loading target of 50 µM. Indeed, decreasing immunogenicity with decreasing incubation time, with immunogenicity matching the 50 µM IMQ standard by 24 h, was observed (Figure 4b). Additionally, the high protein concentration required to deliver 50 µM of IMQ from the bioconjugate showed small but significant immunogenicity on its own and we attribute this to the later time points exceeding the IMQ standards. Overall, this highlights the ability of the bioconjugate to release a biologically relevant substrate, in a time-dependent manner, sustained over several days after in situ labeling with LDNPC (8).

In conclusion, we have demonstrated ligand-directed nitrophenol carbonate (LDNPC) chemistry in a proof-of-concept example utilizing a biotin directing group to modify avidin in situ with a self immolative spacer and imidazoquinoline payload. We have shown, using structural biology, that the modified biotin with appended nitrophenolate, remains a viable ligand (Kₐ = 2.25 x 10⁻⁷ M) for avidin. Additionally, the crystal structure of avidin with the biotin-phenol leaving group (7) demonstrates the abundance of nucleophiles proximal to the biotin binding site with S101 and K111 the two most spatially viable leads for further investigation into the exact mechanism of LDNPC mediated bioconjugation to avidin and subsequent release of payload. At this time, we have qualitatively and quantitatively shown that labeling of the biomolecule occurs and that once labeled, the resulting bioconjugate releases its payload via hydrolysis in a time-dependent manner over the course of 72 h. We believe that this biotin-directed example of LDNPC chemistry will find use in the mature field of avidin-biotin biotechnology where it can add an additional dimension of capability by introducing time-dependent avidin directed substrates. More generally, we have demonstrated proof-of-concept LDNPC chemistry that is a viable technique for the formation of transient in situ bioconjugates. Our lab intends to explore further ligand-protein pairs as well as therapeutic and diagnostic payloads within this framework.

Acknowledgements

Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under Award Number R01CA234115. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Crystallography was supported by a grants from NSF (CHE 1804699) and Murdock Charitable Trust. X-ray data collection used resources of the Advanced Light Source (beamline 5.0.2), which is a DOE Office of Science User Facility under contract no. DE-AC02-05CH11231.

We also acknowledge the NMR facility at Washington State University. The WSU NMR Center equipment is supported by NIH grants RR0631401 and RR12948, NSF grants CHE-9115282 and DBI-9604689, the Murdoch Charitable Trust, and private donors Don and Marianna Matteson.

Keywords: Affinity Labelling  •  Avidin  •  Bioconjugation• Immunochemistry  •  Prodrugs

[1] P. Elzahhar, A. S. F. Belal, F. Elamrawy, N. A. Helal, M. I. Elbayoumi, in Pharmaceutical Nanotechnology (Eds.: V. Weissig, T. Elbayoumi), Springer New York, New York, NY, 2019, pp. 125–182.
[2] I. R. Vlahov, C. P. Leamon, Bioconjugate Chem. 2012, 23, 1357–1369.
