Analytical Methods to Support Design and Optimization of Protein Drug Conjugate: Focusing on Haptoglobin-hemoglobin Complex as a Drug Carrier

Shengsheng Xu

University of Massachusetts Amherst

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ANALYTICAL METHODS TO SUPPORT DESIGN AND OPTMIZATION OF PROTEIN DRUG CONJUGATE: FOCUSING ON HAPTOGLOBIN-HEMOGLOBIN COMPLEX AS A DRUG CARRIER

A Dissertation Presented

by

Shengsheng Xu

Submitted to Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY

May 2017

Department of Chemistry
ANALYTICAL METHODS TO SUPPORT DESIGN AND OPTIMIZATION OF PROTEIN DRUG
CONJUGATE: FOCUSING ON HAPTOGLOBIN-HEMOGLOBIN COMPLEX AS A DRUG CARRIER

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Approved as to style and content by:

_______________________________
Igor A. Kaltashov, Chair

_______________________________
Richard W. Vachet, Member

_______________________________
Stephen J. Eyles, Member

_______________________________
James J. Chambers, Member

_______________________________
Richard W. Vachet, Head

Department of Chemistry
DEDICATION

To my girlfriend Ms. Hongyu Diao and my family
ACKNOWLEDGEMENTS

At first, I would like to express my sincere gratitude to my advisor Dr. Igor Kaltashov for his advice, guidance and continuous support during my Ph.D. career. Each step of my professional growth benefits a lot from his critical thinking and questioning on my research.

I would like to thank the rest of my dissertation committee members: Dr. Richard Vachet, Dr. Stephen Eyles and Dr. James Chambers for their insightful comments, but also for the hard questions which incented me to widen my research from various perspectives.

My sincere thanks also go to Dr. Rinat Abzalimov and Dr. Cedric Bobst for the training and useful discussions. I would like to thank my labmates Dr. Guanbo Wang, Dr. Shunhai Wang, Dr. Son Nguyen, Dr. Khaja Muneeruddin, Dr. Ololade Fatunmbi, Dr. Gregoire Bonvin, Hanwei Zhao, Yunlong Zhao, Jake Pawlowski, Chengfeng Ren, Wenhua Yang and Chendi Niu for their support in my research. I also thank Dr. Sukru Gokhan Elci and Singyuk Hou from Dr. Richard Vachet’s group for the ICP-MS measurement work.

Last but not the least, I would like to thank my parents: Mr. Jianhu Xu and Ms. Yinhua Lu and my girlfriend: Ms. Hongyu Diao for their spiritual support throughout my Ph.D. career.
ABSTRACT

ANALYTICAL METHODS TO SUPPORT DESIGN AND OPTMIZATION OF PROTEIN DRUG CONJUGATE: FOCUSING ON HAPTOGLOBIN-HEMOGLOBIN COMPLEX AS A DRUG CARRIER

MAY 2017

SHENSHENG XU, B.S., WUHAN UNIVERSITY
Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Igor A Kaltashov

Acquired immunodeficiency syndrome (AIDS) remains one of the most serious public health challenges and a significant cause of mortality for certain populations. Despite the large number of antiretrovirals (mostly nucleotide and nucleoside analogs) developed in the past two decades, the inability of small molecule therapeutics to target HIV reservoirs directly creates a significant obstacle to their effective utilization. Indeed, achieving the desired therapeutic effect in the absence of the effective targeted delivery must rely on dosage escalation, which frequently causes severe toxicity. This problem may be solved by conjugation of antiretroviral agents to endogenous proteins (e.g., hemoglobin haptoglobin complex) that are specifically recognized by HIV reservoirs (such as macrophages) for internalization and catabolism. However, conjugation of a large class of antiretroviral agents (acyclic nucleoside phosphonates, such as adefovir) to a protein is challenging due to the rapid decay (including hydrolysis and dimerization) of the activated form of the drug (adefovir phosphonoimidazolide) during transition (ether precipitation) from organic phase to aqueous phase. This work introduces a novel synthetic strategy
which overcomes the instability of the activated form of adefovir by emulating the first step of its metabolic pathway (phosphorylation), making it highly reactive towards primary amine groups of proteins. The effective conjugation of phosphorylated form of adefovir to protein via an imidazolide based intermediate was demonstrated using lysozyme as a model carrier protein. Mass spectrometry (MS) based analytical methods were used to support design and optimization of all those conjugations. Further optimization of adefovir’s conjugation with hemoglobin (Hb), a drug carrier which targets macrophage via haptoglobin (Hp)-CD163 mediated heme scavenging system, was pursued using another novel linker, phosphonoacetate, which allows reactions to be performed at neutral pH with a satisfactory yield. Successful loading of adefovir to Hp, the obligatory partner of Hb for targeted drug delivery, via Hb-Hp binding was demonstrated by MS. Lastly, a new strategy was developed for detecting and quantitating exogenous Hp-Hb complex with high sensitivity in complex biological samples using gallium as a tracer of this protein and inductively coupled plasma mass spectrometry (ICP MS) as a method of detection.
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1. Acyclic nucleoside phosphonates

Acyclic nucleoside phosphonates (ANPs) (e.g., adefovir, tenofovir and cidofovir) are nucleotide analogs which exhibit a broad spectrum of antiviral activities, particularly against DNA virus and retrovirus [1]. Their active metabolites, diphosphorylated ANPs (ANPpp) act as strong competitors of DNA polymerase’s normal substrates [e.g., deoxyadenosine-5’-triphosphate (dATP) and deoxycytidine-5’-triphosphate (dCTP)] and terminate the replication of viral DNA. The common structural attribute of those compounds is a nucleoside attached to an aliphatic chain which contains a phosphonomethyl residue [2]. The methylene bridge between the phosphonate and the rest of nucleoside moiety allows ANPs to resist 5’-nucleotidase catalyzed dephosphorylation which happens frequently on monophosphorylated nucleoside analogs and prevents the conversion to their active metabolites (i.e., nucleoside triphosphate) [3].

1.1. Traditional prodrugs of acyclic nucleoside phosphonates

Acyclic nucleoside phosphonates (ANPs) contain phosphonic acid groups which ionize completely at physiological pH [2]. Those deprotonated compounds become largely impermeable to mucosal and cellular membrane [2]. Prodrugs enable ANPs to overcome the physiological barriers during the delivery by enhancing their bioavailability. Briefly, the phosphonate groups in those nucleotide analogs can be modified by selected protecting groups to improve ANPs’ lipophilicity, altering drugs’ cell and tissue distribution. Pivaloyloxymethyl (POM) and isopropyloxymethyl carbonate (POC) are two typical
protecting groups used in prodrugs of ANPs (e.g., adefovir dipivoxil [4] and tenofovir disoproxil [5]). The attachment of POM or POC with those nucleotide analogs can be performed by using halogeno carbonyloxymethyl derivatives (e.g., POM-Cl/POC-Cl). Those highly reactive compounds can be attacked by deprotonated ANPs in \( N, N' \)-dimethylformamide (DMF), producing POM or POC based prodrugs (see Scheme 1.1.) [6]. Despite the improved bioavailability, those protecting groups in the prodrugs of ANPs undergo rapid degradation during the intestinal transport due to the esterase-mediated cleavage [7, 8]. For example, POMs in adefovir dipivoxil decompose into pivalic acid and formaldehyde, releasing free adefovir with lower ability to penetrate cell membrane [7]. As a result, accumulation of those unprotected drug compounds in kidney causes severe nephrotoxicity which becomes a critical concern in this prodrug’s clinical trials and eventually terminates its application in anti-HIV treatment [9]. The proposed pathology for this renal toxicity is the competition between adefovir’s active metabolites (i.e., adefovir diphosphate) and deoxynucleoside triphosphate in binding with DNA polymerase \( \gamma \) which is responsible for replications of mitochondria DNA [1, 10, 11]. POCs in tenofovir disoproxil also undergo similar degradations, producing unprotected tenofovir, carbon dioxide and formaldehyde [8]. Although tenofovir disoproxil has relatively lower renal toxicity and was approved by US food and drug administration (FDA) for anti-HIV therapy, long term oral administration of this prodrug also leads to patients’ kidney dysfunction [12, 13]. Most recently, an aryloxyphosphonamidate based prodrug of tenofovir, tenofovir alafenamide, was approved by FDA for treatment of HIV infection [14, 15]. The daily dose and maximum concentration in plasma of this prodrug are 25 mg and 16 ng/mL respectively, significantly
lower than those of tenofovir disoproxil: 300 mg/d and 250 ng/mL respectively [14]. Therefore, tenofovir alafenamide has great potential to reduce drug’s kidney exposure and nephrotoxicity in treatment of AIDS. This aryloxyphosphonamidate prodrug is prepared by a different synthetic strategy. Instead of using chlorinated protecting groups, tenofovir was activated by thionyl chloride (SOCl₂), producing highly reactive phosphonyl chloride which can be attacked by the phenol and alanine (see Scheme 1.1.) [6].

1.1.2. Next generation of acyclic nucleoside phosphonates’ prodrug: protein drug conjugate

Although aryloxyphosphonamidate based prodrug reduces tenofovir’s kidney exposure significantly [14], this type of improvement in drug’s pharmacokinetic profiles focuses on alteration of compound’s lipophilicity and fails to enable a site specific delivery which can minimize the toxicity and maximize therapeutic efficacy. Recently, protein-mediated drug delivery has emerged as a promising platform for delivering cytotoxic agents to cancer cells site-selectively. Monoclonal antibodies are selected as carriers of those chemotherapeutics because of their recognition by specific receptors on the membrane of target cells. Until now, two antibody drug conjugates (ADC) (i.e., brentuximab vedotin and trastuzumab emtansine) are approved by FDA for cancer therapy (see details in Figure 1.1.) [16]. This protein-mediated delivery strategy can be also extended to transport of adefovir/tenofovir to cellular reservoirs of HIV. Haptoglobin [17], one of the most abundant plasma proteins, is a promising candidate carrier for ANPs’ targeted drug delivery. The major function of this protein is sequestration of free hemoglobin (Hb) from circulation to avoid possible renal damage and other negative consequences of intravascular
hemolysis [18]. Hb-Hp complexes are processed (catabolized) in macrophages, and their internalization is triggered by binding to a multi-functional receptor CD163 (Figure 1.2). Since macrophages and their progenitors (monocytes) play a prominent role in the establishment of certain types of viral infections (including HIV), virus dissemination, and development of viral reservoirs [19], an ability to deliver anti-viral therapeutics (e.g., adefovir and tenofovir) directly to macrophages (e.g., by conjugating them to Hp, Hb or Hb-Hp) should result in a dramatic improvement of drug efficacy.

As far as we know, ANPs’ conjugation with protein carriers in aqueous solution has not been investigated even though large numbers of small molecule based prodrugs were synthesized in organic solvents. Chlorination by thionyl chloride or oxalyl chloride are frequently used to activate ANP in DMF (see Scheme 1.1.) [6]. The resultant phosphonyl chloride is highly reactive and undergoes attacks by nucleophiles such as phenol and amino acids [15]. However, this activation method is not suitable for ANPs’ conjugation in aqueous solution because both chlorinating agents (i.e., thionyl chloride or oxalyl chloride) and activated form of ANP (i.e. phosphonyl chloride) undergo severe hydrolysis with the presence of water. Another synthetic approach uses N, N’-dicyclohexylcarbodiimide (DCC) to activate ANP, producing phosphonylisourea based intermediate. This type of activation requires long-term stirring at high temperature (80-100°C) to assure a better yield [6, 20]. That harsh reaction condition probably causes severe aggregation of protein carriers during the conjugation. Although there are no examples for ANPs’ conjugation in aqueous solution, activation of ANPs can follow strategies used by nucleoside monophosphate’s conjugation with proteins. For example, the monophosphorylated form of ribavirin, a
nucleoside analog used in treatment of hepatitis C virus infection, was activated by carbonyldiimidazole in DMF and its active intermediate, ribavirin monophosphorimidazolide, was then transferred to aqueous solution to conjugate with hemoglobin [21]. Production of this nucleoside monophosphorimidazolide based intermediate can be also performed in water using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and imidazole [22]. These strategies can be taken into consideration in design of the synthetic protocol for protein/ANP conjugates.

1.2. Analytical techniques to characterize protein drug conjugate

Conjugation of ANPs randomly targets primary amines on the protein surface. The formation of covalent bond between drug’s phosphonate and lysine residues probably eliminates amine’s positive charge and increases negative charges. Those impacts on protein’s charge patterns vary at different conjugation sites and can be evaluated by ion exchange chromatography (IXC). The resin of IXC is modified by either anionic (e.g., carboxylate and sulfonate) or cationic (e.g., amine) functional groups, allowing the retention of negatively or positively charged proteins respectively. The elution of protein analytes can be performed using a buffer with either higher ionic strength or altered pH.

Although IXC enables the separations of protein drug conjugates’ positional isomers based on their charge patterns, it lacks the ability to obtain more details (e.g., mass) about those analytes. Mass spectrometry is a powerful analytical technique which provides sensitive and accurate measurements of ions’ mass-to-charge ratio (m/z) for a variety of biomolecules including protein drug conjugates [23]. For example, drug to protein ratio
(DPR) is an important characteristic of protein drug conjugate, which indicates numbers of payloads. Determination of this ratio relies on accurate mass measurement performed by MS [24, 25]. Since the amino acids sequence of protein carrier and reaction mechanism are well known, expected molecular weights of protein drug conjugates with varying payloads can be easily calculated, and any mismatches with those theoretical masses suggest impurities [26]. In addition, DPR is also a pharmacokinetic parameter which monitors the chemical stability of protein drug conjugates during the delivery. Drug cleavages which occur in plasma probably cause toxicity and need to be evaluated [27].

1.3. Methods for pharmacokinetic study of protein drug conjugates

Pharmacokinetic (PK) of small molecule medicine and protein therapeutic is one of the important factors which decide the drug’s clinical success. Protein therapeutics frequently display unique and unpredictable PK profiles which reflects their differential susceptibility to proteolysis, renal clearance, and interaction with the host’s immune system [26]. Protein drug conjugates may exhibit even more complex PK properties due to their unique molecular structures which incorporate large and small molecule characteristics [28].

1.3.1. Enzyme linked immunosorbent assay (ELISA)

ELISA is a sensitive method widely used to quantitate both proteins and conjugated drugs in ADC samples [27]. Briefly, capture reagents (e.g., antigen) immobilized on solid phase selectively bind with antibodies. Detection reagents, typically horseradish peroxidase (HRP) conjugated anti-human antibody, are added to associate with those analytes and to catalyze oxidation reactions on substrates, resulting in colorful or
fluorescent products for measurement by spectrophotometer [27]. This technique has a few limitations. For example, the concentration measurements of analytes depend on the binding properties between analytes and assay reagents, normally 1:1 binding ratio. Therefore, although this technique can quantitate total antibody and total antibody conjugated drugs in serum/plasma, it fails to differentiate ADCs with varying drug to antibody ratios (DARs) and to provide an average DAR, an important PK property which indicates possible drug release from ADCs in serum/plasma [27]. In addition, this method is not suitable for quantitation of protein carriers which share sequence with their endogenous counterparts. For example, ELISA cannot be applied to quantitate transferrin-CRM107 conjugate due to the abundant interference from endogenous transferrin [29].

1.3.2. Liquid chromatography mass spectrometry (LC-MS)

MS is a powerful technique widely applied in PK study of protein therapeutics. Since protein therapeutics in circulation represent a small fraction of the total plasma proteome, quantitation of those therapeutics using LC-MS requires protein enrichment (e.g., immunocapture) to enhance quantitation sensitivity by increasing analytes concentration and reducing the matrix interference and signal suppression from other proteins in the sample [26, 30]. For accurate and reliable quantitation of protein therapeutics, an isotopically labeled protein or peptide needs to be introduced as the internal standard (IS) to overcome the matrix effect (or ion suppression) during the analysis [31]. IS can be introduced metabolically during cell culture using isotope-enriched medium (e.g., $^{13}$C, $^{15}$N) [32]. This approach effectively avoids errors from downstream sources [33], however,
expression of protein therapeutics with isotope labels in eukaryotic systems is costly and labor-intensive [32]. Comparing with the protein-based isotope labeling, introducing stable isotopes at peptide level during digestion (e.g., enzymatic catalyzed $^{18}$O labeling) becomes a more attractive approach due to its relative ease and universality of labeling [34]. However, this technique fails to make a distinction between an exogenous protein and its endogenous counterpart if the degree of structural similarity between them is very high. An alternative approach to introducing distinct labels to exogenous proteins is to tag metals on protein surface, making it detectable by inductively coupled plasma (ICP) MS [35].

1.3.3. Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS is a powerful technique widely used for element analysis, which relies on high-temperature plasma that breaks down compounds into atoms and eventually ionizes them before transfer to a MS analyzer [36]. This technique is recently expanded to absolute quantitation of protein therapeutics using a metal labeling strategy [36]. Rare-earth elements such as lanthanide are frequently used in metal tags to enable sensitive quantitation of proteins in ICP-MS based analysis due to their high ionization efficiency and low background (e.g., absence of rare earth element in biological samples and less polyatomic interferences). For example, metal-coded affinity tag (MeCAT), a lanthanide-containing reagent which consists of 1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (DOTA) and cysteine-reactive maleimide group (see Figure 1.2.), was developed for ICP-MS based quantitation of monoclonal antibody [37]. This metal tag reagent targets free thiol groups after reducing inter-chain disulfide bonds of antibody,
thereby impacting less on antibody’s confirmation or binding affinity [37]. In addition to protein quantitation, ICP-MS is coupled with laser ablation (LA) to allow the biodistribution analysis of the metal tagged protein therapeutics [36]. This technique uses laser with high-power to ablate the tissue surface, sweeping sample aerosol into ICP-MS [36]. It was applied in the analysis of biodistribution of holmium MeCAT tagged anti-HER2 antibody on breast cancer tissues [38].

1.4. Objectives

ANPs are promising nucleotide analogs used in antiretroviral therapy. Their ionizable phosphonate groups limit drugs’ ability to penetrate cell membrane, resulting in unsatisfactory pharmacokinetic (PK) profiles [39, 40]. Current strategies to improve ANPs’ PK properties focus on tuning their lipophilicity by derivatization. However, delivery of those prodrugs is still not site selective and may cause off-target toxicity. Protein mediated delivery becomes an alternative way to optimize PK parameters of ANP due to protein’s specific recognition by receptors expressed on membrane of target cells. Conjugation of ANPs with those protein carriers is desirable to avoid drug’s dissociation during the transport. However, most of synthetic strategies of ANPs were developed for aprotic solvent based reaction environment. High temperature, high pH and long-term stirring are allowed to ensure a better yield. None of those conditions are suitable for protein. Therefore, it is important to develop novel and robust synthetic strategies to allow the conjugation to be performed in protein friendly environment such as room temperature, neutral pH and aqueous solution. The design and optimization of those synthetic protocols heavily rely on the supports from analytical tools such as
chromatography and mass spectrometry. And this work consists of several projects aiming to use those techniques to assist preparation and characterization of protein/adefovir conjugates as well as tracing the protein based drug carriers. Specifically, the objectives of this dissertation include: (i) Overcoming the hydrolytic liability of a reaction intermediate in production of protein/drug conjugate: conjugation of acyclic nucleoside phosphonate to a model protein carrier (Chapter 2); (ii) Conjugation of antiretroviral drug (adefovir) with hemoglobin and loading it to haptoglobin, an obligatory partner for targeted delivery (Chapter 3); (iii) Evaluation of gallium as a tracer of exogenous hemoglobin-haptoglobin complex in targeted drug delivery application (Chapter 4).
Figure 1.1. Top: brentuximab vedotin (Adcetris®), an anti-CD30 antibody conjugated with MMAE for the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma; Bottom: trastuzumab emtansine (Kadcyla®), an anti-HER2 (human epidermal growth hormone receptor 2) antibody conjugated with DM1 for the treatment of HER2+ metastatic breast cancer.
Figure 1.2. Endosomal routing scenarios encountered by haptoglobin/hemoglobin complexes in macrophages.
Figure 1.3. Structure of MeCAT consisting of DOTA which chelates with lanthanide and maleimide group which targets cysteine.
Scheme 1.1. Mechanism of conjugation using POM-Cl (top) and activation of adefovir using thionyl chloride (bottom).
CHAPTER 2

OVERCOMING THE HYDROLYTIC LABILITY OF A REACTION INTERMEDIATE IN
PRODUCTION OF PROTEIN/DRUG CONJUGATES: CONJUGATION OF AN ACYCLIC
NUCLEOSIDE PHOSPHONATE TO A MODEL CARRIER PROTEIN

2.1. Introduction

Acquired immunodeficiency syndrome (AIDS) remains one of the world’s most
significant public health challenges and a significant cause of death for certain populations
[41]. Despite the relentless search for a cure of HIV infection, presently antiretroviral therapy
(ART) remains the only feasible therapeutic modality that allows many patients to achieve
and maintain near complete suppression of the virus for extended periods of time [42].
However, the success of ART is undermined by the drug resistance [43], particularly in
pediatric populations [44], placing a premium on the continuous expansion of the repertoire
of available ART agents [45]. Unfortunately, a significant fraction of novel antiretroviral drugs
fail to deliver on their promise, as the inability of small molecule therapeutics to target the
HIV reservoirs creates a significant obstacle to their effective utilization. Indeed, achieving a
desired therapeutic effect in the absence of an effective targeted delivery frequently relies
on dosage escalation, causing severe toxicity. For example, an acyclic nucleotide analog
adefovir [46] that was initially developed as anti-HIV therapeutic failed to gain approval for
that indication due to significant nephrotoxicity, despite having demonstrated a clear
antiretroviral benefit [47]. Pharmacokinetic studies demonstrated that over 98% of
unmodified adefovir molecules are excreted with urine during the 24-hour window
following an intravenous injection [39], clearly preventing it from reaching anatomical and cellular viral reservoirs [48].

Far from being unique to adefovir treatment, nephrotoxicity appears to be a common problem for a number of antiretroviral agents [49-52], imposing significant limitations on available therapeutic options. The problem of dosage-dependent toxicity can be alleviated by targeting the ART agents directly to viruses or viral reservoirs [53, 54], and limiting their levels elsewhere in the organism. One particularly attractive opportunity in this regard is presented by haptoglobin, and acute phase plasma protein that irreversibly binds free hemoglobin molecules in circulation and delivers them to macrophages for catabolism [55]. Internalization of the haptoglobin/hemoglobin complexes is mediated by CD163 receptors that are specific to macrophages and other cells of monocyte lineage[56] that harbor HIV virus in AIDS patients. The potential of CD163 to serve as a target molecule for cell-directed therapies has been recognized several years ago [57]. In fact, conjugation of a nucleoside analog ribavirin to hemoglobin had been explored for targeted delivery to CD163-expressing cells [58] with the goal of overcoming the dose-dependent toxicity. While this particular nucleoside analog cannot be used in ART, a strategy can be envisioned where ART agents are conjugated to haptoglobin/hemoglobin complex to achieve targeted delivery to macrophages via CD163-mediated internalization to inhibit reverse transcription of HIV in infected immune cells during early stages of acute infection [3, 59]. A particularly attractive class of agents includes nucleotide reverse transcriptase inhibitors (NtRTIs) incorporating structural features such as phosphonate groups that prevent them from being processed by nucleases [60]. Targeted delivery of such NtRTIs utilizing vehicles capable of traversing
physiological barriers (e.g., transferrin or anti-transferrin receptor antibodies[61]) can also solve the problem of poor accessibility of viral pools in the central nervous system, where cells of monocyte/macrophage lineage (e.g., microglia, perivascular macrophages and meningeal macrophages) harboring HIV are protected by the blood brain barrier [62, 63].

Conjugation of one of the members of the NtRTI family, cidofovir, to a serine/tyrosine dipeptide has been recently demonstrated [64-66]. The phosphonate group of cidofovir readily forms phosphonooester bond with the hydroxyl group of serine in N, N'-dimethylformamide (DMF). However, this conjugate undergoes rapid hydrolysis in aqueous environment at physiological pH with an estimated half-life 0.5-1.4 hours [64, 65], which may not provide an adequate time window for successful delivery of an ART agent to its target site. Furthermore, most carrier proteins are likely to undergo unfolding even at relatively low content of organic solvent, causing aggregation and precipitation during the conjugation process. Therefore, it is highly desirable that the carrier protein/drug conjugation reactions take place in an aqueous environment under conditions that do not compromise the higher order structure of the protein. In this respect, a more attractive scenario would include two steps: chemical activation of the anti-viral agent in organic solvent followed by the transfer of the chemically active intermediate to an aqueous protein solution to produce a conjugate. This scheme was used successfully to produce a ribavirin/hemoglobin conjugate[58] by activating ribavirin with carbonyldiimidazole (CDI) in DMF to produce a reactive phosphorimidazolide intermediate. However, our attempts to adopt this strategy to conjugate NtRTIs (such as
adefovir) to proteins failed due to the extreme instability of the phosphonoimidazolide intermediate compared to its phosphorimidazolide counterpart.

In this work, a modified conjugation strategy is presented which overcomes the problem of the phosphonoimidazolide intermediate instability by chemically modifying adefovir prior to its activation. This initial modification emulates the first step in adefovir’s metabolic pathway (phosphorylation), allowing a phosphorimidazolide intermediate to be produced that is reactive towards primary amines (and, to a lesser extent, hydroxyl groups) on the protein side chains, and at the same time shows remarkable stability vis-à-vis hydrolytic attacks by water. The new conjugation protocol can be used to attach adefovir and other structurally related anti-viral agents to proteins recognized by the cell surface receptors specific to macrophages (such as haptoglobin/hemoglobin complex), enabling targeted drug delivery directly to the cells harboring HIV viruses.

2.2. Experimental

Materials. Adefovir (9-(2-phosphonomethoxyethyl) adenine, PMEA) was purchased from AK Scientific, Inc. (Union City, CA). Lysozyme (Lz), 1, 1′-carbonyldiimidazole (CDI), phosphoric acid (crystalline 99.999% metal trace basis) and imidazole were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) was purchased from Pierce Biotechnology (Rockland, IL). Triethylamine (TEA), N, N′-dimethylformamide (DMF) and anhydrous diethyl ether were purchased from Fisher Scientific (Fair Lawn, NJ). All other solvents and reagents were of analytical grade or higher.
PMEA activation and preparation of conjugates. Preparation of the Lz/PMEA conjugate is shown in Scheme 2.1., 2.2. and 2.3.. PMEA was converted to PMEA phosphonoimidazolide (PMEA-Im) using a modification of the previously published procedure [21]. Briefly, PMEA (15 mg, 0.054 mmol) powder was placed in a 2 mL glass vial. CDI (0.082 mmol, 1.5 equiv) was dissolved in 400 μL DMF/TEA mixture (3:1, v:v). The solution was stirred at room temperature for 1.5 hours, followed by addition of 36 μL of 3.1 M solution of anhydrous phosphoric acid (0.054 mmol, 1 equiv) in DMF. The phosphorylation was performed overnight at room temperature. Afterwards, the reaction solution was placed to cold diethyl ether to precipitate adefovir monophosphate (PMEAp). The cloudy diethyl ether was kept at -20 °C for 2 hours followed by centrifugation, after which the supernatant was decanted. The waxy precipitate at the bottom was re-dissolved in 100 μL distilled water and divided equally in two parts, which were mixed with 400 μL 0.2 M imidazole buffer (pH 6.8) and 400 μL 0.2 M sodium bicarbonate buffer (pH 8.7), respectively. EDC (0.027 mmol, 1 equiv) was added to both reaction buffers and imidazole (0.027 mmol, 1 equiv) was added to the 0.2 M sodium bicarbonate buffer. Both reaction solutions were stirred at room temperature for half an hour. A 50 μL aliquot of the 1 mM lysozyme stock solution was added to each of the reaction buffers (450 μmol), giving a final protein concentration of 100 μM. The final drug-to-protein molar ratio in aqueous solution was around 540:1. Both reaction solutions were stirred at room temperature for 24 hours.
**Characterization of PMEA derived small molecules.** Mass measurements of PMEA derived small molecules were carried out with a QStar-XL (AB-Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer using negative ion mode.

**Characterization of the conjugates.** Mass measurements of Lz/PMEA conjugates were carried out with a QStar-XL (AB-Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer followed their purification by buffer exchange in 100 mM ammonium acetate; ions were produced by electrospray ionization (ESI) using a turbospray source. The conjugates were desalted and denatured via running through C18 column (Waters, Milford, MA) before an off-line MS based characterization. Fragmentation of conjugate ions was carried out by selecting a precursor ion (m/z selection window 1627.0 ± 0.4 u) followed by its collisional activation (50 eV collisional energy). Assessment of the conjugate heterogeneity (profiling of positional isomers) was carried out by introducing an online ion-exchange chromatography (IXC) step prior to ESI MS measurements using a method developed earlier in our laboratory [67, 68]. Briefly, IXC (cation exchange) was performed on Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatograph equipped with a 2.1 mm × 100 mm PolyCAT A (PolyLC Inc., Columbia, MD) weak cation-exchange column (particle size of 5 μm and pore size of 300 Å). Ammonium acetate at pH 7.0 was used for MS-compatible gradient elution (100 mM in mobile phase A and 1 M mobile phase B). The flow rate was maintained at 0.1 mL/min, and the linear gradient ran from 0% to 60% mobile phase B during a 60 min time interval. A Synapt G2Si (Waters, Milford, MA) hybrid quadrupole/time-of-flight mass spectrometer was used for on-line ESI MS detection.

**2.3. Results and discussions**
2.3.1. Preparation of lysozyme/adefovir conjugates.

Our initial attempt to conjugate adefovir to a model protein (lysozyme) using the procedure designed for conjugating a chemically similar drug ribavirin to hemoglobin [58] failed to generate any detectable conjugates despite the presence of 7 primary amines (targets of the conjugation reaction) within the protein. MS analysis of the activated form of adefovir produced in DMF yielded a mass spectrum with a single peak at \( m/z \) 322 (Figure 2.1.) consistent with the notion of the expected product (adefovir phosphonoimidazolide (PMEA-Im), see Scheme 2.1.) being produced at nearly 100% yield. However, the conjugation reaction takes place in the aqueous environment, and addition of even small amount of water to the DMF solution of phosphonoimidazolide (ca. 2.5 % by volume) resulted in a dramatic alteration of the appearance of the mass spectrum following a relatively short (15 min) incubation time period (Figure 2.1.). Although the phosphonoimidazolide ion is still present in the spectrum, the most abundant ionic signal corresponds to a species at \( m/z \) 527; a lower-abundance peak corresponding to unmodified adefovir (\( m/z \) 272) is also present. The mass of ion at \( m/z \) 527 is consistent with the dimeric form of adefovir (linked via pyrophosphonate); this species is formed not only as a result of exposure to water, but also during diethyl ether precipitation (data not shown), an essential step required to remove unconsumed reagents and organic solvents from the chemically activated adefovir prior to its conjugation to a protein.

The re-appearance of the signal of unmodified adefovir (\( m/z \) 272) in the mass spectrum of its activated (phosphonoimidazolide) form exposed to water is likely due to hydrolysis (Scheme 2.1.) facilitated by formation of the transition zwitterion. Indeed, the
methylene bridge (-CH₂-) in the phosphonoamide moiety exerts an electron donating inductive effect on both phosphorus and nitrogen atoms, leading to their basicity increase [69]. As a result, the proton of the hydroxyl group attached to the phosphorus atom is expected to migrate to the nearby basic nitrogen atom of the imidazole ring, forming a transition zwitterion and making imidazole a good leaving group [70]. Formation of this species (with a good leaving group, imidazole) is expected to occur in the phosphonoimidazolide intermediate, with the imidazole group being rapidly replaced by water (or another agent capable of a nucleophilic attack) [70]. This behavior is in contrast to that of phosphorimidazolide which had been demonstrated to be stable to hydrolytic nucleophilic attacks [71], and is likely due to the high basicity of the phosphorus–linked nitrogen atom [70].

The hydrolyzed form (i.e. adefovir) of adefovir phosphonoimidazolide (PMEA-Im) is strongly nucleophilic and can also attack the phosphorus atom in the zwitterionic form of PMEA-Im, leading to formation of a pyrophosphonate-linked PMEA dimer (Scheme 2.1.), following essentially the same mechanism as the one proposed for the hydrolytic attack by a water molecule. The molecular weight of this dimeric product is consistent with the mass of the most abundant ionic species in the mass spectrum of water-exposed PMEA-Im (m/z 527 in Figure 2.1.), lending further credibility to the proposed mechanism.

The anomalous basicity of the nitrogen atom in phosphonoamide bond can be tuned via a variety of ways, and perhaps the most straightforward approach is to modify α-carbon of phosphorus in PMEA with an electron withdrawing group, such as trifluoromethyl [70]. However, the structural change caused by this particular
modification appears to be too significant, and is likely to result in loss of drug’s ability to bind with HIV’s reverse transcriptase. An alternative approach adopted in this work relies on extending phosphonate by converting adefovir to adefovir monophosphate, a strategy informed by the report that nucleoside diphosphorimidazolide is less prone to hydrolysis than nucleoside monophosphorimidazolide, at least in the case of the adenosine 5’-monophosphorimidazolide and 7-methylguanosine 5’-monophosphorimidazolide and their diphosphorimidazolide counterparts [71]. In order to determine if phosphorylation of PMEA will make its imidazolide-based intermediate less susceptible to nucleophilic attack, we first converted PMEA to its monophosphate form (an analog of deoxyadenosine-5’-diphosphate (dADP)) to enhance the stability of the reaction intermediate (Scheme 2.2.). This strategy not only stabilizes the reaction intermediate in aqueous environment without introducing significant alteration to PMEA structure, but also facilitates conversion of PMEA to its active metabolite (PMEApp) via bypassing the first phosphorylation step.

Importantly, replacing imidazole in adefovir phosphonoimidazolide by a chemically active (primary amine-targeting) group from protein surface should not diminish the basicity of phosphorus-linked nitrogen in phosphoamidate moiety and stabilize this linkage [70]. Therefore, PMEA’s conjugation requires efficient linkers to avoid direct reaction between phosphonate group and primary amine from lysine residues. Here, PMEA can be phosphorylated to PMEAp (analog of dADP) [71]. The basicity of nitrogen in phosphoramidate moiety of PMEA monophosphorimidazolide (PMEAp-Im) can be efficiently reduced because the introduced oxygen at α-position of terminal phosphorus
inductively withdraws electrons from nitrogen [69]. Therefore, the formation of transition zwitterion can be inhibited and the intermediate is stabilized. The phosphorylation was carried out adding anhydrous phosphoric acid DMF solution in PMEA-Im. The ESI mass spectrum of reaction mixture acquired at negative ion mode indicates that the major product (i.e. PMEAp m/z=352.2) coexists with hydrolytic product and diphosphorylated product (i.e. PMEApp m/z=432.2) (see Figure 2.1.). To reduce loss of efficient component (PMEAp) during the transfer from DMF to water, PMEAp was directly precipitated in cold diethyl ether rather than activated by CDI again in DMF. The major advantage of this step is that PMEAp has lower solubility in cold diethyl ether (non-polar solvent) than PMEAp-Im, due to its relative higher polarity.

The phosphorylated form of adefovir (PMEAp) extracted from DMF was redissolved in 50 mM pH 6.8 imidazole-HCl aqueous buffer, followed by addition of EDC to induce activation. This reaction produces an O-acylisourea intermediate, which is known to be rapidly attacked by imidazole groups [22], a trait that was exploited in our work to conjugate PMEA to a protein (Scheme 2.3.). The “on-demand” formation of PMEAp-Im in the presence of the protein greatly reduces non-productive reaction channels (e.g., hydrolysis of this intermediate by water molecules), while allowing the nucleophilic attacks by the primary amines (lysine side chains) to take place, leading to the conjugate formation. While the unconsumed EDC is usually quenched prior to mixing with protein solutions with 2-mercaptoethanol, we chose to avoid this step, as the presence of this reagent in the protein solution would almost certainly induce reduction and/or rearrangement (scrambling) of disulfide bonds. The downside of keeping EDC in solution
is the occurrence of side reactions, although they can be suppressed by selecting appropriate reaction conditions (*vide infra*).

### 2.3.2. Characterization of the lysozyme/adefovir conjugates.

ESI mass spectra of Lz/PMEA conjugates prepared at pH 6.8 and pH 8.7 are shown in Figure 2.2. The ionic species present in each mass spectrum display narrow charge state distributions, and the average extent of multiple charging is consistent with compact (natively oxidized) conformations in solution [72]. Both spectra contain signals of intact (unmodified) Lz and a 1:1 conjugate. In addition, the mass spectrum of the conjugate produced under mildly basic (pH 8.7) conditions contains ionic signals corresponding to conjugates with 2:1 stoichiometry, as well as a range of other products. The mass analysis of these modified Lz species reveals two types of chemical modification (addition of 71.1 and 128.2 Da, consistent with reactions of residual EDC with the protein molecule [20]), as well as their combinations with each other and with PMEA/Lz conjugates. Specifically, modification of carboxylic groups of aspartic and/or glutamic acid residues is likely to give rise to unstable O-acylisourea intermediates, which are then converted to stable N-acylurea products via the intramolecular O-to-N acyl rearrangement in the presence of excess EDC [73] (see Scheme 2.4.). No dead-end products are observed in the mass spectrum of Lz/PMEA conjugate produced at pH 6.8, indicating that the unstable O-acylisourea intermediates undergo quick hydrolysis prior to acyl rearrangement at neutral pH. Therefore, even though both the conjugation reaction yield and the average drug loading are noticeably lower when the reaction is carried out at neutral pH, these
conditions are still preferable to mildly basic pH, as the products are much less heterogeneous.

2.3.3. Further evaluation of heterogeneity of lysozyme/adaefovir conjugates using online ion exchange chromatography mass spectrometry (IEC/MS).

Although the mass spectrum of Lz/PMEA conjugates produced at neutral pH reveals only a single species (in addition to the intact unmodified protein), the sample may contain several isobaric species that differ from each other by the location of the conjugation site. In order to evaluate the structural heterogeneity of this sample with respect to the possible presence of several positional isomers, it was analyzed by ion exchange chromatography with on-line detection by ESI MS. This technique was recently developed in our laboratory [67] and successfully applied for characterization of samples as complex as PEGylated glycoproteins subjected to stress conditions [68]. Both total ion and UV absorption chromatograms (UV data is shown in Appendix F) of the Lz/PMEA conjugates show a series of peaks, and the on-line MS detection provides evidence that all species eluting before 55 minutes have masses consistent with 1:1 conjugation stoichiometry (Figure 2.3.). Integration of the intensities of the Lz/PMEA conjugates’ peaks in UV absorption chromatogram suggests that the yield of the conjugation reaction is 23%, which is reasonably close compared to the 27% yield obtained by integrating ion peaks in the mass spectrum of the reaction mixture (Figure 2.2.).

PMEAp conjugation to the protein at the lysine side chain not only eliminates the positive charges on the ε-amine group of this residue, but also introduces negative charges (phosphonate and phosphate groups of PMEAp). Although the total number of
charges is the same for all isomers, the unique position of the PMEAp group on the surface of the protein will have a profound effect on the surface charge density pattern. The charge density is an important determinant of the protein retention on the ion exchange resin [74]; therefore, it should not be surprising that IXC allow a number of isomeric conjugates to be resolved (Figure 2.3). What is surprising, however, is the fact that the number of resolved isobaric conjugates exceeds the total number of amines (six \( \varepsilon \)-amines at lysine side chains and one \( \alpha \)-amine at the N-terminus of the protein). One plausible explanation for the large number of the observed isobaric species could be the occurrence of protein deamidation during the conjugation process. Deamidation is a common non-enzymatic post-translational modification (PTM), which frequently (and inadvertently) occurs during a variety of protein handling steps [75], making it a serious nuisance in biopharmaceutical analysis. Deamidation results in a mass increase of only 1 Da, which is nearly impossible to detect when the protein mass is large; however, in our case a one-mass unit shift in isotopic distributions of the Lz/PMEA conjugate should be readily detected because of the relatively low mass of the latter. No such shift was detected for either of the species eluting prior to 55 min. in IXC shown in Figure 2.3, suggesting that deamidation does not take place under the conditions chosen to produce the conjugates (pH 6.8).

In order to understand what could have contributed to the unexpectedly large number of isomeric 1:1 Lz/PMEA conjugates, collisionally activated dissociation (CAD) of the mass-selected Lz/PMEA ions was carried out and the mass spectra of the resulting fragments (Figure 2.4.) was analyzed. CAD of the Lz/PMEA ion at +9 charge state (m/z
1627.6) gives rise to a relatively small number of fragment ions. Singly charged fragment ions are observed in the low-\(m/z\) region of the mass spectrum (\(m/z\) 274 and 354) and their complementary fragments at high \(m/z\) region (\(m/z\) 1597.3, 1786.8 and 1796.8). The most abundant of the two low-mass fragments (\(m/z\) 274) corresponds to a \((\text{PMEA}+\text{H})^+\), produced by the cleavage of P-O bond in the phosphate group of the conjugate. The complementary ion gives is represented in the CAD mass spectrum with an abundant peak at \(m/z\) 1797 (charge state +8), and a fragment ion with the same mass but different charge state (+9) is observed at \(m/z\) 1597 (corresponding to the neutral loss of PMEA from the precursor ion). The presence of these fragment ions in the CAD mass spectrum of the Lz/PMEA conjugate is not surprising, since the phosphoester bonds are known to be labile in the gas phase and undergo dissociation prior to the cleavage of the polypeptide amide bonds [76]. What is surprising, however, is the presence of the second low-\(m/z\) peak in the CAD mass spectrum (a singly charged species at \(m/z\) 354). This mass corresponds to a \((\text{PMEAp}+\text{H})^+\) ion, which can only be produced upon fragmentation of the Lz/PMEA conjugate if PMEAp is attached to the protein via hydroxyl-bearing side chain, such as serine or threonine, as opposed to a “conventional” linkage via the amino group of lysine side chains and the polypeptide N-terminus. Phosphoserine peptides are known to undergo fragmentation upon collisional activation to lose 98 Da mass (equivalent to a phosphoric acid compound) and to produce dehydroalanine via \(\beta\)-elimination [76]. Gas phase fragmentation of serine linked PMEAp probably follows the same mechanism, producing a singly charged PMEAp.
The existence of the PMEAp/protein conjugates where the pro-drug is linked to the polypeptide chain via side chains with hydroxyl-group not only explains the appearance of the CAD spectrum (Figure 2.4.), but also accounts for the anomalously high number of chromatographic peaks in the XIC for the 1:1 conjugate, which exceeds the number of primary amine groups in the protein (Figure 2.3.). In retrospect, conjugation at Ser (and possibly Thr) residues using the amine-targeting chemistry presented in this work may not be that surprising, because their side chains become nucleophilic upon deprotonation which is induced by surrounding basic residues (e.g., lysine and arginine) and is probably solution pH independent, while neutral pH limits the fraction of primary amine that can participate in the attack of activated form of PMEAp. From the practical standpoint, the ability of hydroxyl groups to compete with primary amines as conjugation sites may actually prove beneficial vis-à-vis designing affective means of targeted delivery to macrophages, as no solvent accessible Ser and Thr residues of haptoglobin are known to participate in binding to either hemoglobin or CD163 (the cell-surface receptor that enables internalization of the Hp/Hb complexes by macrophages) via hydrogen bonding or Van der Waals interaction, while 5 Lys residues (K262, K291, K297, K345 and K379) are known to participate in such interactions [77-81].

The significant degree of structural heterogeneity of the 1:1 PMEAp/protein conjugate produced in this work using a model protein provides important lessons vis-à-vis production of adefovir conjugates with real carrier proteins (such as hemoglobin or haptoglobin). Indeed, a very large fraction of hemoglobin (αβ dimer) and haptoglobin surface (14 % and 13 %, respectively) are localized within the binding interface when the
hemoglobin/haptoglobin complex is formed (the surface area buried at the interface was calculated by subtracting the sum of solvent accessible areas of each protein component alone from that for the complex and dividing the result by two, crystal structure of human hemoglobin/haptoglobin complex (pdb: 4X0L) was used for the calculation of buried area). These interface regions incorporate 6 residues (i.e., K291, K297, K345 and K379 on Hp; V1 and K99 on Hb(α)) whose side chains or N-terminal are targeted by the conjugation reaction, which include 11% of all surface primary amines. In addition, some Ser or Thr residues located in these binding areas might undergo deprotonation and become nucleophilic, and the participation of those residues in conjugation largely depends on their solvent accessibility and local electrostatic environment. Since neither hemoglobin nor haptoglobin alone can effectively interact with the CD163 receptor (a critical step in internalization of these proteins by macrophages), it appears that covalent attachment of PMEAp to any of these side chains will have a strong negative impact on the effectiveness of targeted delivery by compromising the integrity/stability of the hemoglobin/haptoglobin complex. In the absence of the effective chemical means to direct the conjugation reaction away from these critical sites, it would seem prudent to produce the conjugates using the hemoglobin/haptoglobin complex as a starting material, instead of either hemoglobin or haptoglobin alone. Formation of a stable complex prior to the conjugation procedure will shield the critical Lys, Ser and Thr side chains from the adefovir monophosphorimidazolide. In the absence of such “natural” protection during the conjugation step, due care should be exercised to ensure the ability of the conjugates
to associate with the physiological partner thereby enabling the receptor recognition and conjugate internalization by the targeted cells.

### 2.4. Conclusions

In this work, we introduced a robust synthetic strategy to overcome the instability of adefovir phosphonoimidazolide by emulating the first step (i.e. phosphorylation) of adefovir metabolic pathway and used this strategy to demonstrate successful conjugation of this anti-viral drug with a model protein. The phosphorylation of adefovir allows the conversion of α-position functional group of terminal phosphorus from methylene (-CH₂-) to oxygen, altering the electron density of phosphorus linked nitrogen and reducing its basicity in imidazolide based intermediate. This modification efficiently inhibits the migration of proton from phosphorus to its adjacent nitrogen and a formation of transition zwitterion, which frequently occurs in phosphonoimidazolide based intermediates and causes their hydrolytic instability. Mass spectrometry was used to monitor the outcomes of individual steps in the adefovir’s activation, allowing us to understand why the classical conjugation chemistry failed in this drug. Use of mass spectrometry was also critical in selecting the optimal conditions for the coupling of adefovir monophosphate to the model protein, as the conditions favoring higher conjugation yield (and higher drug-to-protein loading ratio) were shown to also lead to formation to a large amount of dead-end (hydrolyzed) cross-links and mixed products. A reasonable compromise between modest conjugation yields (27%) and product purity (almost exclusively 1:1 conjugate) was found; however, the conjugate was shown to exhibit significant structural micro-heterogeneity due to distribution of the conjugation
sites among 12 protein site chains. This exceeds the number of primary amines (the intended targets of the conjugation reaction), suggesting involvement of other group(s) in conjugation. Fragmentation patterns of the conjugates activated by collisions in the gas phase provide unequivocal evidence that primary alcohols (side chains of Ser and Thr) also participate in conjugation reactions. The novel synthetic strategy developed in this work can be applied to other anti-viral drugs (such as tenofovir, which exhibits a structural template similar to adefovir, including the presence of a phosphonate group, as well as other members of acyclic nucleoside phosphonate family). A work is currently underway in our laboratory to apply the new synthetic strategy to conjugate adefovir to a hemoglobin/haptoglobin complex, enabling targeted delivery of this drug directly to cellular reservoirs of HIV.
Figure 2.1. Mass spectra of PMEA-Im in DMF (brown) and DMF/water (blue) and PMEAp in DMF (black) acquired at negative ion mode. Ion peaks: m/z 322, 527, 272, 352 and 432 represent PMEA-Im, PMEA dimer, PMEA, PMEAp and PMEApp respectively.
Figure 2.2. ESI mass spectra of the products of Lz conjugation with PMEAp carried out at pH 8.7 (red trace) and 6.8 (blue trace). The inset shows a zoomed view of ions with charge state +9: the base peak at m/z 1590.4 corresponds to intact Lz; mass shift of 335 Da corresponds to conjugation of a single PMEAp to the protein, while additions of 71, 128 and 199 Da represent Lz’s N-acyl-N-3-ethyl carbamic acid derivative, N-acyl-N-3-dimethylaminopropyl carbamic acid derivative and their combination respectively (see Scheme 2.4. for more structural details).
Figure 2.3. Total ion chromatogram (black trace) of Lz/PMEA conjugate produced at pH 6.8 and extracted ion chromatograms for the unmodified protein (blue), 1:1 conjugate (purple) and 2:1 conjugate (red) plotted with different magnification factors (the m/z values indicate the ions selected for plotting each XIC). The inset shows zoomed views of ion peaks at charge state +8 from the mass spectra averaged across the elution windows as indicated on the panel (peaks labeled with stars correspond to sodium adducts).
Figure 2.4. Mass spectrum of fragment ions produced by collisional activation of 1:1 Lz/PMEA conjugate (m/z 1627.6). The diagrams in the inset show structure of observed fragment ions (fragments that can originate from precursors where PMEAp is conjugated either to primary amines or alcohol hydroxyls are labeled in red, while the fragments that can originate exclusively from precursors where PMEAp is conjugated to alcohol hydroxyls are labeled in blue).
Scheme 2.1. Mechanisms of PMEA activation with CDI (top), formation of PMEA-Im transition zwitterion and hydrolysis (middle) and formation of PMEA dimer (bottom).
Scheme 2.2. Mechanism of phosphorylation of PMEA.
Scheme 2.3. Mechanisms of activation of PMEAp with EDC in aqueous solution (top) and the conjugation of PMEAp with primary amine (middle) and hydroxyl groups (bottom).
Scheme 2.4. Mechanisms of formation of N-acyl-N-3-dimethylaminopropyl carbamic acid (NDCA) and N-acyl-N-3-ethyl carbamic acid (NECA).
CHAPTER 3

CONJUGATION OF ANTIRETROVIRAL DRUG (ADEFOVIR) WITH HEMOGLOBIN AND LOADING IT TO HAPTOGLOBIN, AN OBLIGATORY PARTNER FOR TARGETED DRUG DELIVERY

3.1. Introduction

Adefovir, namely 9-[2-(phosphonomethoxy)ethyl]adenine (PMEA), is a promising antiretroviral drug which was developed for anti-HIV therapy [3]. Its active metabolite, adefovir diphosphate [PMEApp, analog of deoxyadenosine-5’-triphosphate (dATP)], is able to incorporate with HIV’s reverse transcriptase and terminate the viral complementary DNA’s replication [82]. To increase the bioavailability of this drug, two pivaloyloxymethyl (POM) groups are conjugated with PMEA’s phosphonate via phosphonoester bonds to produce its prodrug adefovir dipivoxil [bis(pivaloyloxymethyl)-PMEA, bis(POM)-PMEA]. However, clinical trials of this prodrug in treating HIV infected patients were discontinued due to kidney toxicity [12, 83]. Although POM facilitates PMEA’s intestinal transport, it undergoes rapid chemical and enzymatic degradation during this process as well as in plasma, resulting in PMEA and mono(POM)-PMEA with reduced cell permeability [7, 84]. Those hydrolytic products of bis(POM)-PMEA accumulate in kidney via renal clearance instead of reaching therapeutic targets, causing nephrotoxicity. Alternatively, POM was replaced by 1-aryl-1,3,,-propanyl in a PMEA’s prodrug called pradefovir to increase the liver accumulation and to reduce kidney exposure of PMEA in treatment for another viral infection [i.e. hepatitis B virus (HBV)] [85]. This prodrug maintains high stability in plasma because it can be only cleaved by liver specific enzyme [85]. However, the clinical trials of
pradefovir in anti-HBV treatment was terminated owing to adverse results in non-clinical carcinogenicity studies [86]. Alkoxyalkyl monoesters such as hexadecyloxypropyl (HDP) are another type of protecting groups for drugs to increase their intracellular concentration taking advantages of lipid uptake pathway [87]. Conjugation of HDP with PMEA was demonstrated to improve PMEA’s efficacy against HIV [87, 88]. However, HDP-PMEA was found to have much lower half maximum cytotoxic concentration (CC₅₀): 60 nM than that of PMEA (CC₅₀: 157 µM), indicating a significant increase of toxicity after the conjugation [88].

Protein mediated targeted delivery has great potential to improve the site-selectivity of PMEA’s transport in vivo. Conjugation of PMEA with a model protein carrier, lysozyme, has been recently accomplished using a phosphate linker (see Chapter 2). In this synthetic strategy, phosphorylation of PMEA overcomes the instability of the activated form of PMEA (adefovir phosphonoimidazolide, PMEA-Im), making it reactive towards primary amine groups on protein. However, this conjugation requires long term stirring (more than 24 hrs) and basic reaction pH (pH 8.7) which probably introduce a risk of protein aggregation and post-translational modifications (e.g., deamidation), causing loss of material and safety issues in drug application [89]. In this work, we developed a novel phosphonoacetate linker for PMEA’s conjugation with protein carrier. This new strategy was applied on hemoglobin (Hb), a promising drug carrier which binds haptoglobin (Hp) with high affinity and targets macrophage via CD163 mediated endocytosis [21]. This linker allows effective conjugation of PMEA with protein to be performed under neutral pH and with significant reduced reaction time. Although a few byproducts are introduced in this
reaction, Hb/PMEA conjugates remain the main species. The successful loading of PMEA on Hp·Hb complex via Hp and Hb’ association indicates that these conjugates are suitable for further evaluation of their cellular uptake and bioactivity.

3.2. Experimental

Materials. Adefovir was purchased from AK Scientific, Inc. (Union City, CA). 1,1’-carbonyldiimidazole (CDI), phosphonoacetic acid (PA), dithiothreitol (DTT) and human hemoglobin were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). N-hydroxysulfosuccinimide (Sulfo-NHS), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Pierce Biotechnology (Rockland, IL). Triethylamine (TEA), trifluoroacetic acid (TFA), formic acid, N, N’-dimethylformamide (DMF), anhydrous diethyl ether and acetonitrile were purchased from Fisher Scientific (Fair Lawn, NJ). Human haptoglobin phenotype 1-1 (Hp) was purchased from Athens Research & Technology (Athens, GA).

Synthesis of adefovir phosphonoacetate (PMEAPA) and preparation of hemoglobin/adefovir conjugates (Hb/PMEA conjugates). The scheme for preparation of Hb/PMEA conjugates is shown in Scheme 3.1.. Adefovir was converted to adefovir phosphonoacetate (PMEAPA) based on the modified protocol used in adefovir’s phosphorylation (see Scheme 2.1. and 2.2.). Briefly, adefovir (10 mg, 0.036 mmol) was placed in a 2 mL glass vial, followed by addition of 300 μL 0.24 M CDI (0.072 mmol, 2 equiv) solution which contains 75% (v/v) DMF and 25% (v/v) TEA. The cloudy reaction mixture was stirred at room temperature until it becomes clear. 10 mg (0.072 mmol, 2 equiv) phosphonoacetic acid powder was added to this solution and the mixture was stirred at
room temperature overnight. Afterwards, the reaction solution was placed in cold diethyl ether to precipitate PMEAp. The cloudy diethyl ether was kept at -20 °C for 2 hours prior to centrifugation. The white precipitates at the bottom of the centrifuge tube were re-dissolved into 300 μL 0.2 M phosphate buffer (pH 7.5), followed by addition of EDC (0.11 mmol, 1.5 equiv) and sulfo-NHS (0.11 mmol, 1.5 equiv) and stirring at room temperature for 20 min. 2 μL 500 mM DTT was added in solution to quench extra EDC before mixing with 700 μL 5.3 mg/mL Hb solution (0.2 M phosphate buffer, pH 7.5). The concentration of Hb and PMEAp to Hb ratio were calculated to be 3.7 mg/mL [equivalent to 57 µM Hb] and 500:1 respectively. The reaction solution was stirred for 30 min prior to addition of 2 μL 1 M glycine to quench unreacted sulfo-NHS ester based compounds. The quenching step lasted 20 min.

**Liquid chromatography mass spectrometry (LC/MS) based characterization of Hb/PMEA conjugates.** The reaction mixtures were analyzed with reverse phase LC/MS using an Agilent 1100 HPLC (Agilent, Santa Clara, CA) coupled with a Bruker SolariX 7.0T Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA). Hb (α)/PMEA conjugates and Hb (β)/PMEA conjugates were separated using a Waters BioSuite™ C18 column (100Å, 3 μm, 2.1×50 mm) at a flow rate of 0.25 mL/min with the elution shown in Table 3.1. Mobile phase A and B were water and acetonitrile respectively, containing 0.1% formic acid and 0.02% TFA. The UV absorbance was measured at 280 nm.

**Native electrospray mass spectrometry (native ESI-MS) based characterization of Hp·Hb/PMEA conjugates complex.** Off-line native ESI-MS analysis of Hp·Hb/PMEA
conjugates complex was carried out using a QStar-XL (AB-Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer equipped with a nanospray source. The desalted Hb/PMEA conjugates were incubated with human Hp (4:1 molar ratio) at 4°C for 2 hours. Afterwards, Hp-Hb/PMEA conjugates complex was isolated by Agilent 1100 HPLC (Agilent, Santa Clara, CA) equipped with a TSKgel G3000SWxl size exclusion column (Tosoh, Tokyo, Japan). The separation was performed using 150 mM ammonium acetate solution as mobile phase at a flow rate of 0.8 mL/min. The UV absorbance was measured at 280 nm.

3.3. Results and discussions

3.3.1. Preparation of hemoglobin/adeovir conjugates.

A phosphate linker had been previously reported to enable PMEA’s conjugation with a model protein carrier, lysozyme (see Chapter 2). Phosphorylation of PMEA allows the formation of stable phosphoramid bond with primary amine groups from protein surface. However, this reaction using phosphorimidazolide based intermediates (i.e. the activated form of adefovir monophosphate: adefovir monophosphorimidazolide) requires long term stirring and basic pH to assure a better yield (see Chapter 2). Stirring Hb for a few days probably introduces a risk of aggregation and precipitation during the conjugation due to its lack of disulfide bonds to maintain compact conformation. We selected phosphonoacetate as a linker to improve the reaction efficiency of protein PMEA conjugation. The advantage of this linker is that it provides not only phosphonate which acts as strong nucleophile to attack adefovir phosphonoidazole (PMEA-Im, the activated form of adefovir) but also carboxylate group which targets primary amine
groups with the activation by EDC/sulfo-NHS [20]. Compared to phosphorimidazolide based reaction intermediates frequently used in nucleotide’s bioconjugation, sulfo-NHS ester intermediates have much higher efficiency in amide bond formation [20-22]. The ESI mass spectrum of reaction mixture (after addition of phosphonoacetate) acquired in negative ion mode indicates the production of adefovir phosphonoacetate (measured m/z equals 394.0, very close to calculated m/z of PMEAp: 394.03) (see Figure 3.1.). PMEAp was then activated by EDC/sulfo-NHS to conjugate with Hb in 0.2 M phosphate buffer, pH 7.5 [20].

3.3.2. Characterization of hemoglobin/adeovir conjugates.

Sulfo-NHS esters of PMEAp form amide bonds with primary amine groups from solvent accessible lysine residues/N-terminus on Hb effectively. Each conjugation of PMEAp will result in 377.19 Da mass addition on either Hb α-chain or β-chain. Since PMEAp compounds are not purified prior to mixing with Hb (CDI, DMF and TEA are removed by ether precipitation), some impurities originated from its production might induce side reactions during the conjugation with Hb. PMEA and PMEA dimer are probably two small molecule byproducts which coexist with PMEAp after ether precipitation. However, neither of them is able to participate in the reaction with Hb because PMEA cannot form stable phosphonoamide bond with primary amine group and PMEA dimer has strong steric hindrance which prevents it being activated by EDC/sulfo-NHS [70]. Excess phosphonoacetate added to ensure high yield of PMEAp’s production might be a concern about introducing protein byproducts because this compound contains carboxylate group which can be activated by EDC/sulfo-NHS. The mass addition on Hb caused by
phosphonoacetate conjugation is calculated to be 122.02 Da. Reverse phase LC/MS was used to characterize hemoglobin α-chain PMEA conjugates [Hb (α)/PMEA conjugates] and hemoglobin β-chain PMEA conjugates [Hb (β)/PMEA conjugates]. ESI mass spectrum averaged from 29.8 min to 37.8 min (see Figure 3.2.) indicates that it consists of Hb (α)/PMEA conjugates and very few Hb (β)/PMEA conjugates (see Figure 3.3.). The most abundant peak (m/z 862.24 charge state 18+) in this mass spectrum indicates a mass shift 376.92 Da on Hb (α) (calculated mass addition 377.19 Da), corresponding to Hb (α)/PMEA conjugates with 1:1 drug to protein ratio (DPR) (see Table 3.2.). The calculation of Hb (α) average DPR was carried out with the assumption that ion intensities (I) of protein drug conjugate (PDC) species observed in mass spectrum correlate to their real quantities. The percentage (%) of PDCs with same DPR was calculated using this equation:

$$\text{Percentage (\%)} = \frac{\sum I_{\text{PDCs with same DPR}}}{\sum I_{\text{total PDC}}} (1)$$

Average DPR of Hb (α) was calculated to be 1.4 using the equation as follows [24]:

$$\text{Average DPR} = \sum (\text{percentage of PDCs with same DPR} \times \text{number of loaded drugs}) (2)$$

As anticipated, there are a few ion peaks representing Hb (α)/phosphonoacetate conjugates (see Figure 3.3.).

ESI mass spectrum averaged from 38.7 min to 47 min of reverse phase chromatogram (see Figure 3.2.) indicates that it contains Hb (α)/PMEA conjugates and Hb (β)/PMEA conjugates (see Figure 3.4.). The most abundant ion peak (m/z 882.14 charge state 19+) in a region (m/z 880-930) of this mass spectrum represents Hb (β) conjugated with two PMEAp (mass addition 377.19×2 Da) and one phosphonoacetate (mass addition 122.02 Da) (see Table 3.2.). The ion peak (m/z 883.82 charge state 19+) is assigned as the oxidized form.
of the previously mentioned Hb (β)/PMEA conjugate (m/z 882.14 charge state 19+) due to a 31.92 Da mass addition (accuracy: 4.7 ppm). The presence of this peak suggests the oxidation of Cys residues into their sulfenic acid derivatives with a 32 Da mass addition [90, 91]. This modification originates from the commercial Hb which is already oxidized [91]. The average DPR for Hb (β) was calculated to be 1.8 using the same method applied on Hb (α)-PMEA conjugates. Therefore, the average DPR for intact Hb (α2β2) can be calculated to be 6.4 if conjugation does not interrupt formation of Hb tetramer.

3.3.3. Characterization of haptoglobin·hemoglobin/adefovir conjugates complex using native ESI-MS.

There are two equilibria: (α2β2)⇌2αβ and 2αβ⇌2α+2β in Hb’s solution at neural pH [92], allowing the access of reaction intermediates (sulfo-NHS ester of PMEAPA) to the binding areas. The crystal structure of human Hb (pdb: 2DN2) indicates that the binding interface of two αβ dimers in Hb includes two amino acid residues (i.e. αV1 and αK99) which contain primary amines [93]. Those residues probably participate in the conjugation due to the exposed interface. Based on the crystal structure of human Hp·Hb complex (pdb: 4X0L), Hb αV1 and αK99 display direct contact with corresponding residues (A382, V383 for αV1 and A288, F290 for αK99) in Hp via hydrogen bonding and Van der Waals interactions (see Figure 3.5.) [80]. Therefore, PMEAPA’s incorporation at these two sites (i.e. Hb αV1 and αK99) probably interrupt the binding between Hp and Hb, lowering the actual drug loading on Hp·Hb complex. Native electrospray ionization mass spectrometry (native ESI MS) provides an elegant way to evaluate this impact because it preserves non-covalent interactions in gas phase. This approach can be used to determine
the DPR at Hp·Hb complex level. The Hp·Hb/PMEA conjugates complex was prepared by incubating Hp with Hb-PMEA conjugate \((\alpha^*\beta^*)_2\) at 1:4 molar ratio. The resultant protein complex was fractionated by size exclusion chromatography (SEC) prior to analysis by native ESI MS. The off-line ESI mass spectrum of the earlier eluting species (a fraction collected within the 8.3-9.7 min elution window) (see Figure 3.6.) acquired under near-native condition indicates that the ionic species confined to a narrow \(m/z\) window 5000 - 6000 corresponds to Hp·Hb/PMEA conjugates complex \([Hp\cdot(\alpha^*\beta^*)_2]\) (see red trace in Figure 3.7.). Its mass is calculated as 161 kDa based on \(m/z\) values for the centroids of the four most abundant peaks \((m/z\ 5196\pm 60, \text{ charge state } 31^+;\ m/z\ 5369\pm 90, \text{ charge state } 30^+;\ m/z\ 5552\pm 90, \text{ charge state } 29^+;\ m/z\ 5755\pm 50, \text{ charge state } 28^+\). This mass is 3.6 kDa higher than the theoretical mass for the Hp·Hb/PMEA conjugates complex (calculated as 157,345.4 Da for the major glycoform of Hp and assuming 6.4 PMEApapa payloads on the sequences of all proteins involved), and a mismatch of this magnitude is to be expected given the extensive solvation of large protein ions generated by native ESI [94, 95]. Meanwhile, the off-line ESI mass spectrum of SEC fractionated Hp·Hb complex was acquired under the exact same condition used by Hp·Hb/PMEA conjugates complex (see black trace in Figure 3.7.). The mass of Hp·Hb complex was calculated to be 159.7 kDa using \(m/z\) value of centroids of most abundant peaks, 1.3 kDa lower than that of Hp·Hb/PMEA conjugates complex. That mass difference is equivalent to 3.4 average drug to Hp·Hb complex ratio (mass addition of each PMEApapa conjugation: 377.19 Da). This mismatch of DPRs between protein complex and tetrameric Hb indicates that not all of Hb/PMEA conjugates participate in binding with Hp. It is possible that conjugations occur
in or near the association interface (e.g., Hb αV1 and αK99) between Hb and Hp and affect their binding. Since the binding interface between Hb and Hp highly overlaps with that of two αβ dimers, these conjugations probably impact the formation of Hb tetramers. Therefore, the actual DPR for intact Hb can be adjusted to be 3.4. Since those equilibria of Hb in aqueous solution are pH dependent [93], a slight increase of solution pH favors the formation of Hb tetramer. Under this circumstance, the binding interface of two αβ dimers is buried, inaccessible to those reaction intermediates. As a result, this association region on Hb can be preserved for binding with Hp. Despite those binding interruption, other lysine residues on Hb (α) and Hb (β) are located far away from Hb’s interaction regions with Hp, therefore, drug conjugations at those sites probably exert no impact on loading adefovir to Hp via Hb and Hp binding.

After successful loading of adefovir to Hp-Hb complex, it is necessary to evaluate whether this targeted delivery system improves the pharmacokinetic profiles of the drug. Quantitation of exogenous Hp-Hb complexes carrying adefovir compounds in biological matrices can be performed by tagging these protein carriers with no-cognate metals (e.g., gallium) and measuring the latter’s concentration (will be converted to Hp-Hb complexes’ concentration based on tagging stoichiometry) via inductively coupled plasma mass spectrometry (ICP-MS) [59]. However, since drug release might occur before reaching therapeutic targets due to enzymatic catalyzed or chemically induced cleavages, the DPR for Hp-Hb complex may vary during the transport, affecting the accuracy of conjugated drug’s quantitation. To solve this problem, an attractive ruthenium (Ru) based metal tag designed for conjugated PMEApA is currently being pursued in our laboratory. Dual metal
labeling approach allows ICP-MS to measure concentrations of gallium and ruthenium in biological samples simultaneously, which will be converted to concentrations of Hp·Hb complexes and their payloads respectively. This method can be used to monitor drug release of Hb/adenovir conjugates before its arrival at target sites or after their internalization [96].

3.4. Conclusion

The development of small molecule drugs is frequently impeded because of their unsatisfactory pharmacokinetic profiles [97]. The off-target toxicity of those drugs is one of main causes for their clinical failures [98]. For example, adefovir is a promising antiretroviral drug designed for inhibition of HIV’s reverse transcription. However, its prodrug (i.e. adefovir dipivoxil)’s clinical trials in anti-HIV therapy was terminated owing to nephrotoxicity. This problem can be solved by targeting adefovir to HIV’s cellular reservoirs (e.g., macrophage, monocyte) by protein based carriers to reduce drug’s exposure to kidney. Conjugation of adefovir with a model carrier protein, lysozyme, was carried out using a phosphate linker. Despite the success, this reaction requires long term stirring and basic pH to assure a satisfactory drug loading, increasing a risk of protein aggregation and post-translational modifications (e.g., deamidation). In order to solve this problem, we developed a phosphonoacetate linker to enable efficient and robust conjugation of adefovir with Hb, a real protein carrier. This reaction using the new linker can be performed at physiological pH with less than one hour stirring, reducing the loss and damage of Hb. The average DPRs for Hb α- and β-chain drug conjugates after 30 min reaction were determined by mass spectrometry to be 1.4 and 1.8 respectively. Although
linker (i.e. phosphonoacetate) derived byproducts were observed in the mass spectrum, Hb-PMEA conjugates remain the major components of the protein mixtures. The actual number of PMEA loading on Hp-Hb complex was determined to be 3.4 by native ESI based measurements, lower than the assumed DPR 6.4 (based on DPRs for Hb α- and β-chains). This result indicates that conjugation may take place in the binding interface between Hb and Hp, impacting their association. This problem can be solved by burying the interfaces between two αβ dimers during the reaction by a slightly increased pH which favors the formation of Hb tetramer.
<table>
<thead>
<tr>
<th>Elution time, min</th>
<th>Mobile phase A (0.1% formic acid, 0.02% TFA in water), %</th>
<th>Mobile phase B (0.1% formic acid, 0.02% TFA in acetonitrile), %</th>
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</thead>
<tbody>
<tr>
<td>0</td>
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**Table 3.1.** Elution gradient for Hb/PMEA conjugates’ isolation by reverse phase chromatography.
<table>
<thead>
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<th>average mass, Da</th>
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**Table 3.2.** Measured masses and calculated masses for Hb/PMEA conjugates shown in Figure 3.3. and 3.4. For Hb (α)/PMEA conjugates and Hb (β)/PMEA conjugates, the number of PA equals that of PMEA in their notation. For phosphonoacetate derived impurities, the number of PA differs from that of PMEA in their notation.
Figure 3.1. Zoomed view of ESI mass spectrum of adefovir phosphonoacetate (PMEAp) acquired under negative ion mode.
Figure 3.2. Reverse phase chromatogram (brown) of Hb/PMEA conjugates acquired at wavelength 280 nm (from online reverse phase LC-MS analysis).
Figure 3.3. Top: ESI mass spectrum averaged from 29.8 min-38.5 min of reverse phase chromatogram (see Figure 3.2.) for separation of Hb/PMEA conjugates. Bottom: zoomed view of above mass spectrum. For Hb (α)/PMEA conjugates, the number of PA equals that of PMEA in their notation. For phosphonoacetate derived impurities, the number of PA differs from that of PMEA in their notation.
Figure 3.4. Top: ESI mass spectrum averaged from 38.7 min-47 min of reverse phase chromatogram (see Figure 3.3.) for separation of Hb/PMEA conjugates. Bottom: zoomed view of above mass spectrum. For Hb (β)/PMEA conjugates, the number of PA equals that of PMEA in their notation. For phosphonoacetate derived impurities, the number of PA differs from that of PMEA in their notation.
Figure 3.5. Selected binding interface between human Hb (α) (yellow) and Hp (purple) (extracted from pdb: 4X0L), αV1 involves in hydrogen bonding interaction with A382 (4.2 Å) and V383 (2.8 Å) in Hp (Top). αK99 engage the hydrogen bonding interaction with A288 (3.5 Å) in Hp (Bottom).
Figure 3.6. Size exclusion chromatogram of Hp/Hb/PMEA conjugates mixture acquired at 280 nm.
Figure 3.7. Off-line ESI mass spectra of SEC fractionated Hp·Hb/PMEA conjugates complex (red) and Hp·Hb complex (black) (collected over 8.3-9.7 min, see Figure 3.6) acquired at near native condition. The insert is the zoomed view of charge state 30+ and 29+ of Hp·Hb/PMEA conjugates complex (red) and Hp·Hb complex (black). The asterisk label means PMEA conjugated protein.
Scheme 3.1. Reaction scheme of Hb adefovir conjugation using phosphonoacetate linker. Formation of transition zwitterion in PMEA-Im probably occurs due to the electron-donating effect from methylene group which is adjacent to phosphonimidazole moiety. In this transition zwitterion, imidazole becomes a good leaving group which can be replaced by nucleophiles easily.
CHAPTER 4

EVALUATION OF GALLIUM AS A TRACER OF EXOGENOUS HEMOGLOBIN-
HAPTOGLOBIN COMPLEXES FOR TARGETED DRUG DELIVERY APPLICATIONS

4.1. Introduction

Haptoglobin (Hp) is an acute phase plasma protein, whose raison d’être is sequestration of free hemoglobin (Hb) in circulation to avoid possible renal damage and other negative physiological consequences of intravascular hemolysis [18]. In the past, interest in Hp was caused primarily by its obvious ability to attenuate efficiency of oxygen transport by Hb-based blood substitutes [99], as well as its involvement in iron acquisition by several pathogens [100]. More recently, better understanding of the Hp-mediated pathway of Hb clearance [55, 101] has provided strong indications that this protein may also be used for targeted drug delivery, taking advantage of the fact that Hb·Hp complexes are processed (catabolized) in macrophages [17, 102]. Since macrophages and their progenitors (monocytes) play a prominent role in establishing certain types of viral infections (including human immunodeficiency virus, HIV), virus dissemination, and development of viral reservoirs [103], the ability to deliver anti-viral therapeutics directly to macrophages (e.g., by conjugating them to either Hp or Hb·Hp complexes) should result in a dramatic improvement of the drug efficacy. Another high value target for such a strategy might be the hepatitis C virus (HCV), since there is evidence that resident liver macrophages are infected by and support replication of HCV [104]. In addition to viral infections, a similar strategy can be envisioned as a way to design novel therapies against
certain types of cancers (most notably acute myeloid leukemia), as the monocyte/macrophage lineage specificity of CD163 (cell-surface receptor that recognizes Hb-Hp complexes and assists their internalization [17, 56]) expression is preserved beyond malignant transformation [105, 106]. The specificity of targeted cytotoxin delivery in this case would be particularly beneficial, as Hp-mediated drug delivery specifically to the CD163-expressing cells will be limited to monocytes and macrophages and spare normal stem cells in the bone marrow [105].

As is the case with all drug delivery strategies utilizing transport proteins for targeted delivery, successful design of therapies based on Hp as a delivery vehicle (e.g., Hp-cytotoxin or Hp-antiviral conjugates) will critically depend on the ability to trace the drug/protein conjugate within the organism following its administration to obtain detailed and reliable pharmacokinetics and pharmacodynamics information [26, 107, 108]. While the protein quantitation strategies are now well-established, a unique challenge for reliable and sensitive localization and quantitation of protein therapeutics based on or mimicking abundant endogenous proteins (such as Hp) arises from the fact that such measurements are carried out on the varying (and often unpredictable) background of endogenous molecules whose structure is identical to that of the exogenous (administered) proteins. This makes it highly problematic to use classic techniques of protein quantitation, such as enzyme-linked immunosorbent assay (ELISA) [109, 110], which remains one of the most popular and sensitive techniques in the field [111, 112]. Recently, mass spectrometry (MS) emerged as a powerful tool for protein quantitation in complex biological matrices, enabling highly sensitive and selective
measurements both in the field of proteomics [113] and in a variety of pharmacokinetic studies [30, 114, 115]. Most of these applications rely on stable isotope labeling of proteins or their fragments as a means of quantitation [116]. However, because most cost-effective labeling strategies introduce the isotopic label during the proteolytic step [117], they frequently fail to make a distinction between an exogenous protein and its endogenous counterpart if the degree of structural similarity between them is very high. Such a distinction can be made if the isotopic label is introduced at the whole protein level (e.g., during the expression of the exogenous protein), but this usually results in a dramatic increase of the production costs.

An alternative approach to introducing distinct labels to exogenous proteins that can be readily identified by MS takes advantage of the possibility to attach a metal tag to the protein surface, making it detectable by inductively coupled plasma (ICP) MS [35]. A very important advantage of this approach is the high sensitivity afforded by ICP MS measurements and the possibility to select a non-cognate metal tag that would have minimal spectral interferences. However, an obvious drawback of these strategies is their reliance on chemical modification of the protein surface, which may alter its properties, including interactions with physiological partners and therapeutic targets. Recently, we introduced a solution to this problem that can be used when the exogenous protein is a metalloprotein (e.g., ferro-protein transferrin used to deliver drugs either to malignant cells or across physiological barriers [118]), where the cognate metal is replaced with a non-cognate one without altering protein conformation or compromising the receptor recognition. For example, replacing Fe$^{3+}$ with In$^{3+}$ in transferrin allows this protein to be
detected with high sensitivity in blood and tissue homogenates of animals using ICP MS, and its distribution maps to be obtained by imaging with laser ablation ICP MS [119].

In this work we extend this approach to Hp, which is not a metalloprotein. This is done by substituting Fe$^{3+}$-bound protoporphyrin IX (heme) with the protoporphyrin IX molecule that contains a non-cognate metal (gallium) within Hp’s counterpart, Hb prior to forming the Hp·Hb complex (which is the molecular entity recognized by the cell-surface receptor). Despite relatively low stability of Ga-substituted Hb (Hb$^{Ga}$), it binds readily to Hp, with the resulting Hp·Hb$^{Ga}$ complex remaining remarkably stable under physiological conditions. The non-cognate metal allows this complex to be readily detected in serum samples at levels well below 10 nM, which makes it suitable for pharmacokinetic studies.

4.2. Experimental

*Materials.* Hp phenotype 1-1 was purchased from Athens Research & Technology (Athens, GA), and human Hb was purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Gallium (III)-protoporphyrin IX (Ga-PP) was purchased from Frontier Scientific (Logan, UT). Gallium, plasma standard solution (10,000 mg/L) was purchased from Alfa Aesar (Haverhill, MA). Nitric acid (69.2% aqueous solution) and hydrogen peroxide (31.4% aqueous solution) were purchased from Fisher Scientific (Fair Lawn, NJ). Human serum samples were provided by the Department of Kinesiology, University of Massachusetts-Amherst (Amherst, MA). All other reagents and solvents used in this work were of analytical grade or higher.
Methods. The apo-form of human Hb was prepared using a modified acidic acetone precipitation method described in detail elsewhere [120]. Briefly, cold (4 °C) aqueous solution of 200 µM Hb in 150 mM ammonium acetate was infused into cold (0 °C) 2 M HCl acetone solution (mixing ratio 1:50), followed by vigorous mixing for 5 min. The fluffy colorless precipitate was collected by pipette, centrifuged at 4 °C and lyophilized. The lyophilized apo-Hb was dissolved in 10 mM ammonium acetate solution to a final concentration of 0.42 mg/mL and placed on ice for at least 1 hour, followed by the addition of 1 mM Ga-PP methanol solution at a 37:1 ratio (v:v). The molar ratio of Ga-PP to globin monomers was estimated to be 1:1. This solution was incubated on ice for 1 hour to produce Hb^Ga, followed by ESI MS and/or UV/Vis absorption analyses at room temperature. The Hp·Hb^Ga complexes were produced by adding 44 µM solution of Hp in 150 mM ammonium acetate to the ice-cold Hb^Ga solution at a mixing ratio of 1:13 (v:v), corresponding to an 8:1 molar ratio of Hb^Ga globins (monomers) to Hp.

A Nanodrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) was used to measure the UV-Vis absorption spectra of Hb^Ga and Hp·Hb^Ga and evaluate their stability at 37 °C by monitoring the decay rates of their Soret bands within the time interval ranging from 0.5 to 24 hrs. The measured intensities of the Soret bands were fitted to exponential decay curves to estimate the half-life values. SEC fractionation of Hp·Hb^Ga was carried out using Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatograph equipped with a TSKgel G3000SWxl (Tosoh, Tokyo, Japan) column. A 150 mM ammonium acetate solution was used as mobile phase in the separation at a flow rate of 0.8 mL/min. The absorbance was measured at 280 nm and 415 nm. SEC fractions were manually
collected and characterized by native electrospray ionization (ESI) MS with a QStar-XL (AB/Sciex, Toronto, Canada) hybrid quadrupole/time-of-flight mass spectrometer equipped with a nano-spray source.

Human serum spiked with Hp·Hb\(^{\text{Ga}}\) was prepared using the metal-labeled protein solution, in which the total protein concentration was determined by UV absorption measurements at 280 nm using the molar absorptivity (165,700 M\(^{-1}\)·cm\(^{-1}\)) calculated based on the sequence of both Hp and Hb. The detection of Ga in the spiked serum samples was carried out using NexION 300X (PerkinElmer, Waltham, MA) ICP mass spectrometer following its overnight digestion with 49% HNO\(_3\) and 7% H\(_2\)O\(_2\) (by volume) at 37 °C. The kinetic energy discrimination (KED) mode was used to reduce polyatomic interference. The ICP mass spectra were obtained for both the spiked serum sample and the blank (Ga-free serum) by scanning the quadrupole in the \(m/z\) range of 50-150 u.

4.3. Results and discussion

4.3.1. Rationale for selecting gallium as a metal tracer.

Hp is not a metalloprotein, but its binding partner Hb is. Since their association is very strong [121], and the Hp·Hb complex is in fact the molecular entity recognized by CD163 on the macrophage surface (an obligatory first step in its internalization), Hb appears to be an excellent choice for introducing a metal tag that can be used to trace the entire complex. Although the cognate metal (iron) is obviously unfit to be a good tracer (due to its abundance in living organisms), substitution of this metal with a non-cognate one will produce a good tag as long as it does not disrupt (i) the heme interaction with each of the
globin chains (both α and β), and (ii) the association of Hp with the reconstituted Hb. There is a wide variety of non-ferrous metals that can be complexed with protoporphyrin, some of them having physiological significance [122]. However, gallium appears the best substitute for the cognate metal due to the filled d-shell, very close size match (0.62 Å vs. 0.65 Å for Fe³⁺) and its trivalency (even though heme-bound iron exists in the ferrous state inside erythrocytes, hemolysis results in its rapid oxidation to Fe³⁺, leading to formation of met-hemoglobin, which is the form interacting with Hp in circulation). In fact, Ga-substituted protoporphyrin (Ga-PP) is commonly considered a good model to study the heme-globin interaction [123]. Furthermore, gallium is not an essential metal and, unlike most of its neighbors in the periodic table, it is not present in the human body at levels exceeding 0.2 ppb [124] (unless the subjects had chronic exposure to this metal [125]), which should minimize interferences in ICP MS and LA-ICP MS measurements.

4.3.2. Production of HbGa and evaluation of its stability.

A recent study by Pinter et al. demonstrated that Ga-PP can be readily incorporated in myoglobin, a protein that is highly homologous to the Hb α- and β-chains, although the Ga-PP reconstituted protein remained stable only at low temperatures [123]. Incorporation of Ga-PP into Hb in our work was also carried out at low temperature. The correct placement of the prosthetic group within the polypeptide chains was indirectly verified by UV-Vis absorption spectroscopy (Figure 4.1.), which shows a significant red shift of the B-band of Ga-PP (from 406 nm to 415 nm), consistent with the formation of the Soret band; a very similar Ga-PP “insertion signature” was previously reported for myoglobin [123]. A more detailed examination of the composition of the products of Hb
reconstitution with Ga-PP was carried out using native ESI MS, which revealed \((\alpha^{Ga}\beta^{Ga})_2\) as a species giving rise to the most abundant ionic signal (Figure 4.2.). The general appearance of this mass spectrum is very close to that previously reported for commercial ferro-Hb, \(Hb^{Fe}\) [126, 127]. In addition to the tetrameric species, both dimers and monomers are observed, alongside some heme-deficient species (labeled with open circles in Figure 4.2.). Some of these Ga-PP deficient species (i.e., ions lacking a prosthetic group) may be produced by gas phase dissociation, as we note the presence of ions in the low \(m/z\) region of the mass spectrum whose masses and isotopic distribution allow them to be unequivocally identified as singly charged Ga-PP (see the inset in Figure 4.2). These ions are likely to be produced in the gas phase (heme loss is a very common fragmentation pathway for all globins), but it cannot be ruled out that at least some heme-deficient species are present in solution as well. Indeed, the presence of heme-deficient assemblies was previously observed even for \(Hb^{Fe}\) [126], and was ascribed to the presence of oxidized \(\beta\)-chains in the commercial Hb samples which have diminished ability to retain the heme group [127] and significantly enhanced flexibility [128].

Unlike \(Hb^{Fe}\), \(Hb^{Ga}\) was observed to precipitate even at 4 OC, which manifested itself by clouding of the protein solution, followed by formation of visible solid precipitates. One possible explanation for this increased aggregation propensity is that the negatively charged Ga-PP in solution may electrostatically repel the negative \(\beta\)-globin in which \(\text{Cys}^{93}\) (\(pK_a = 8.2\)) is oxidized to cysteine sulfenic acid (\(pK_a = 2\)) or sulfonic acid (\(pK_a = -3\)) [129]. This would exclude partial solvent for \(\beta\)-globins and expose hydrophobic residues, triggering nucleation and irreversible aggregation [130]. The rate of \(Hb^{Ga}\) loss in solution
at physiologically relevant temperature (37 °C) was measured by monitoring the intensity of the Soret band using SEC-purified HbGa as a starting material (Figure 4.3.). A very dramatic decrease of the band intensity is observed within few hours (compare the red and pink traces in Figure 4.3. corresponding to measurements taken 2 hours apart). The rate of the Soret band decay in HbGa sample is consistent with the half-life of this protein being only 2 hours, indicating its high vulnerability under conditions similar to those encountered in circulation.

Hp is known to exert a significant stabilizing effect on proteins it binds, and for that reason is frequently referred to as an “extracellular chaperon” [131, 132]. Since the therapeutic action of Hp/drug (or Hb/drug) conjugate could be exerted only within the context of Hp-Hb complex, possible stabilization of HbGa by Hp would be very relevant vis-à-vis ensuring the reliability of the measurements by minimizing or indeed eliminating the loss of the protein due to aggregation. In order to evaluate the stability of HbGa in complex with Hp, a mixture of the two proteins was incubated at 4 °C followed by SEC fractionation (Figure 4.4.). Both SEC fractions exhibited absorbance at 415 nm, and were assigned as Hp-HbGa (elution window 8.9-9.8 min) and free HbGa (elution window 12.5-14 min); this assignment was confirmed by native ESI MS analysis of both fractions (vide infra). Monitoring the intensity of the Soret band for the early-eluting species (assigned as Hp-HbGa complex) over a one-day period following the fraction collection revealed a markedly increased stability compared to free HbGa. In fact, the measured rate of the Soret band decay was consistent with the half-life of ca. 24 hours. This time window appears to be more than adequate for the measurements of biodistribution of Hp-based...
drugs targeting macrophages, as the physiological clearance of Hp·Hb occurs on a significantly shorter time scale [133].

4.3.3. Characterization of Hp·Hb\textsuperscript{Ga}.

The SEC fractions of the Hb\textsuperscript{Ga}/Hp mixture (1.4 mg/mL and 0.27 mg/mL, respectively) incubated at 4 °C overnight were also analyzed by native ESI MS. The off-line ESI mass spectrum of the earlier eluting species (a fraction collected within the 8.9 – 9.8 min elution window) acquired under near-native condition (blue trace in Figure 4.4., bottom) contains contributions from several ionic species. The ionic signal of the major contributing species is confined to a narrow m/z window 5000 – 6000, and its mass is calculated as 157.7 kDa based on m/z values for the centroids of the three most abundant peaks. This mass is 2.7 kDa higher than the theoretical mass for the Hp·(α\textsuperscript{Ga}β\textsuperscript{Ga})\textsubscript{2} complex (calculated as 154,984.6 Da for the major glycoform of Hp and assuming no modification to the sequences of all proteins involved), and a mismatch of this magnitude is to be expected given the extensive solvation of large protein ions generated by native ESI [94, 95]. Indeed, increasing collisional activation of ions in the ESI interface region by stepping up the declustering potential resulted in a noticeable shift of these peaks towards lower m/z values (consistent with the notion of the partial loss of small molecules from the solvation shell of the surviving complex ion), although this also increased the efficiency of other dissociation channels.

A cluster of low-abundance peaks at high m/z (> 6,000 u) corresponds to the products of gas-phase dissociation of Hp·(α\textsuperscript{Ga}β\textsuperscript{Ga})\textsubscript{2} ions proceeding via a loss of a single globin chain (the complementary fragments populate the low m/z region of the mass spectrum (<
This dissociation channel (asymmetric charge partitioning, where a single highly charged polypeptide chain is ejected from the complex ion), is usually a preferred dissociation channel of protein complexes in the gas phase [134, 135]. Lastly, a group of lower-abundance ions populate a narrow \( m/z \) region of the mass spectrum (4,500-5,000 \( u \)) and partially overlaps with the ionic signal of \( \text{Hp} \cdot (\alpha^\text{Ga} \beta^\text{Ga})_2 \). The masses of these species are consistent with unsaturated Hp/Hb complex (\textit{i.e.}, \( \text{Hp} \cdot \alpha^\text{Ga} \beta^\text{Ga} \)). Since the extent of multiple charging of these species is nearly the same as that of \( \text{Hp} \cdot (\alpha^\text{Ga} \beta^\text{Ga})_2 \), it seems highly unlikely that these ions represent products of gas phase dissociation. Even though in some cases asymmetric charge partitioning is not the only channel of gas phase dissociation of protein assemblies [136], transition from \( \text{Hp} \cdot (\alpha^\text{Ga} \beta^\text{Ga})_2 \) (charge states +27 through +30) to \( \text{Hp} \cdot \alpha^\text{Ga} \beta^\text{Ga} \) (charge states +27 and +28) in the gas phase would require removal of hemoglobin dimer \( \alpha^\text{Ga} \beta^\text{Ga} \) carrying very few charges from the multiply charged assembly, a process that appears to be thermodynamically unfavorable [135]. Although the solution-phase origin of the observed \( \text{Hp} \cdot \alpha^\text{Ga} \beta^\text{Ga} \) species might seem to contradict the observation that the ionic signal intensity ratio \( \text{Hp} \cdot (\alpha^\text{Ga} \beta^\text{Ga})_2 / \text{Hp} \cdot \alpha^\text{Ga} \beta^\text{Ga} \) decreases at higher collisional activation, this is likely to be a consequence of \( \text{Hp} \cdot (\alpha^\text{Ga} \beta^\text{Ga})_2 \) ions being more prone to gas phase dissociation (and suffering greater population loss when the collisional energy is increased).

While it might seem puzzling that the Hp/Hb\( ^\text{Ga} \) complexes of different stoichiometries co-elute in SEC, one must remember that Hp has an extended, dumbbell-shaped conformation, and binding of hemoglobin does not result in a noticeable increase of its hydrodynamic radius [78]. A very modest increase of the extent of multiple charging in
native ESI MS upon Hp association with either $\alpha^{\text{Fe}}\beta^{\text{Fe}}$ (as reported in [94]) or $\alpha^{\text{Ga}}\beta^{\text{Ga}}$ (as seen in Figure 4.4.) is also consistent with a relatively insignificant increase of the physical size of the macromolecule [137]. Therefore, it should not be surprising that $\text{Hp} \cdot (\alpha^{\text{Ga}}\beta^{\text{Ga}})^2$ and $\text{Hp} \cdot \alpha^{\text{Ga}}\beta^{\text{Ga}}$ cannot be separated by SEC. The practical ramifications of this (as related to the ultimate goal of our work) are quite significant, as the presence of the partially unsaturated Hp/Hb complex in the Ga-labeled sample means that the Ga/Hp molar ratio is less than the presumed ratio of 4:1. The magnitude of the correction can be estimated from the ionic signal intensity ratio $\text{Hp} \cdot (\alpha^{\text{Ga}}\beta^{\text{Ga}})^2 / \text{Hp} \cdot \alpha^{\text{Ga}}\beta^{\text{Ga}}$ in the native ESI mass spectrum acquired under mild conditions in the ESI interface region (such as that shown in Figure 4.4.). Even though the relative ion current intensity of a particular species cannot be simply equated to its fractional concentration in solution [138], the correlation could be reasonably close, especially at lower protein concentrations [139]. Based on these considerations, we evaluate the $\text{Hp} \cdot (\alpha^{\text{Ga}}\beta^{\text{Ga}})^2 / \text{Hp} \cdot \alpha^{\text{Ga}}\beta^{\text{Ga}}$ concentration ratio in solution as 3.3 :1, which corresponds to the Ga/Hp labeling ratio of 3.5 :1.

4.3.4. ICP-MS detection of Hp·Hb$^{\text{Ga}}$ in human serum.

Knowing the Ga/Hp labeling ratio provides an opportunity to determine the concentration of this protein (or protein/drug conjugates) in complex biological matrices by measuring the levels of Ga. Ga can be readily detected in a variety of biological matrices using ICP MS, although both of its stable isotopes are known to have spectral interferences, such as $^{138}\text{Ba}^{2+}$ for $^{69}\text{Ga}^+$, and $^{40}\text{Ar}^{31}\text{P}^+$ and $^{35}\text{Cl}^{36}\text{Ar}^+$ for $^{71}\text{Ga}^+$ [140, 141]. Figure 4.5. shows a relevant region of the ICP mass spectrum of a blank (human serum that had not been doped with Ga in any form), where low-abundance ionic signal is
observed at both m/z 69 and 71, even though the measurements were carried out in the kinetic energy discrimination (KED) mode to reduce polyatomic interferences. Spiking the serum sample with Hp/HbGa (final concentration of Hp in the sample 8.4 nM) resulted in a noticeable increase of the ionic signal at both m/z values (see the red-filled curve in Figure 4.5.). The magnitude of the signal intensity increase exceeds an order of magnitude, suggesting that Ga can be successfully used as a tracer in pharmacokinetic studies of Hp-based therapeutics (the protein therapeutics serum concentration range that needs to be accessible to measurements is estimated to span from sub-μM to low-nM levels [142]).

Carrying out the measurements of Ga concentration in a common “hopping mode” (where only the ionic currents of isotopes of interest are measured, such as 69Ga, 71Ga, and the internal standards), as opposed to the continuous quadrupole-scanning mode (shown in Figure 4.5.) will result in further increase of the measurement sensitivity. This will allow exogenous Hp to be detected not only in circulation at concentrations that are significantly below typical levels of the endogenous protein in human plasma [143], but also in various tissues despite unfavorable tissue/serum ratios.

The goal of the present work was to develop a method of detecting and measuring exogenous Hp in complex biological matrices (such as blood plasma), with the subsequent aim of extending this approach to include Hp-based antiviral therapies. One particularly attractive target currently pursued in our laboratory is Hp conjugated to analogs of adefovir [46]. The latter is a small-molecule medicine that was initially designed as an HIV treatment, but the clinical studies showed that absent targeted delivery to macrophages, therapeutically active doses of adefovir are unsafe for patients. As a result, its clinical
applications are now limited to the low-dose format to treat a lower value target, hepatitis B virus [144]. Conjugation of adefovir to the Hp/Hb complex appears to be a reasonable strategy to achieve the desired therapeutic outcome with a lower drug dosage by virtue of targeted delivery. Quantitation of adefovir/Hp/Hb conjugates in biological matrices could be also carried out with high specificity and sensitivity using Ga as a tag by introducing a slight modification to the procedure described in the present work. In this case, Ga/adefovir ratio can be determined by measuring the Ga/P ratio in the conjugate sample by ICP MS (despite interferences from $^{14}$N$^{16}$O$^{1}$$H^{+}$ and $^{15}$N$^{16}$O$^{+}$ [145], ICP MS allows phosphorus to be monitored with a detection limit in the sub-ppb range [146]). Lastly, the sensitivity of detection and quantitation of exogenous Hp or Hp/drug conjugates based on metal tags may be further improved by exploring the utility of other metals beyond gallium, since protoporphyrin IX can be substituted with a variety of non-ferric metals [147].

4.4. Conclusions

Biopharmaceuticals remain one of the fastest growing sectors in modern medicine, but their continued success hinges upon the ability to provide detailed and reliable characterization of these complex therapeutic agents and their behavior in vivo. Collecting pharmacokinetics data is a particularly challenging task for several protein drug candidates, especially those that are structurally very similar or indeed identical to endogenous proteins, e.g. those that enable access to specific target sites. Hp is one of such proteins, as it can uniquely access macrophages, a trait that can be taken advantage of for the purpose of targeted drug delivery in a variety of antiviral strategies. Tracing
exogenous Hp or Hp/Hb complexes in organisms can be very challenging, since these measurements have to be carried out on the background of abundant (an varying) endogenous Hp. We address this problem by substituting iron-bound heme group with the gallium-bound protoporphyrin IX in Hb prior to complexing it with Hp. While Ga-labeled free Hb appears to be only marginally stable, the Hp/Hb\textsuperscript{Ga} complex displays remarkable stability on a time scale relevant for pharmacokinetic measurements. Gallium is a non-essential metal and is not present in humans (absent any environmental or workplace exposure to Ga-containing materials), which makes it a convenient tag that is suitable for detection and quantitation of exogenous Hp/Hb\textsuperscript{Ga} complexes in biological matrices using ICP MS as a method of detection. Despite several spectral interferences, Ga can be detected with sensitivity that is more than adequate for pharmacokinetic studies of Hp-based therapeutics.
Figure 4.1. UV-Vis spectra of a water/methanol solution of Ga-PP (purple), and aqueous solutions of Hb\textsuperscript{Ga} (red) and Hb\textsuperscript{Fe} (brown)
Figure 4.2. Native ESI mass spectrum of a 5 μM solution of Hb\(^{Ga}\) (human Hb reconstituted with Ga-PP) in 150 mM ammonium acetate acquired 60 min. after the incubation. Open circles indicate hemoglobin species lacking one Ga-PP group (i.e., tetramers with three Ga-PP groups and dimers with a single Ga-PP group). The inset shows a low \(m/z\) range of the mass spectrum containing the ionic signal of Ga-PP.
Figure 4.3. UV-Vis absorption spectra of Hb\textsuperscript{Ga} (red and pink) and Hp-Hb\textsuperscript{Ga} (blue and teal); the delay before the acquisition of the second spectrum in each pair was two hours. The insert shows the evolution of the intensity of the Soret band in each sample over a 24 hour time period.
Figure 4.4. Top: Size exclusion chromatogram of Hp and Hb\textsuperscript{Ga} mixture acquired at two different wavelengths (280 nm, purple, and 415 nm, brown). Bottom: off-line ESI mass spectra of SEC fractions acquired over 9-10 min (blue) and 13-14 min (red) time windows. The shaded areas in the mass spectrum of the early-eluting fraction contain gas phase fragment ions produced via asymmetric charge partitioning. Open circles indicate hemoglobin dimers with a single Ga-PP group.
Figure 4.5. Zoomed views of full-scan ICP mass spectra of human serum before (blue trace) and after (red trace) addition of exogenous Hp-Hb$_{Ga}$ complex (the final concentration of the protein in the sample is 8.4 nM).
CHAPTER 5

DISSERTATION SUMMARY AND FUTURE DIRECTION

5.1. Dissertation summary

Proteins have emerged as promising carriers for small molecule drugs to improve their pharmacokinetic profiles in recent years [148-150]. This protein mediated delivery strategy heavily relies on the development of conjugation chemistry which are robust to both protein and small molecule compounds. Traditional synthetic strategies designed for a nucleotide reverse transcriptase inhibitor’s prodrug (adefovir dipivoxil which causes severe nephrotoxicity in anti-HIV treatment) select aprotic solvents (e.g., DMF) as reaction media, allowing the use of highly reactive chlorinating agents which is water-sensitive and high temperature which probably damages proteins. In this dissertation, we developed a novel and robust synthetic strategy to enable adefovir’s conjugation with protein to be performed in aqueous solution. Adefovir compounds were phosphorylated prior to reaction with carriers to avoid direct activation of their phosphonate groups by carbonyldiimidazole, which produces hydrolytically unstable intermediate, adefovir phosphonoimidazolide. The activated form of adefovir monophosphate (i.e. adefovir monophosphorimidazolide) was much more stable than its non-phosphorylated counterpart and was highly reactive towards primary amines and, to less extent, hydroxyl groups on the lysozyme surface. Lysozyme/adefovir conjugates were successfully produced using this novel synthetic strategy and were characterized by mass spectrometry based analysis. This is the first time that protein based prodrug of acyclic nucleoside phosphonate has been prepared in aqueous solution.
The synthetic strategy was further optimized by introducing phosphonoacetate as a linker to provide more robust reaction environment for protein carriers. This novel linker was applied to the conjugation of adefovir with hemoglobin, a potential protein carrier which targets CD163 receptors on macrophages with the assistance of haptoglobin. Phosphonoacetate provides not only phosphonate group which acts as a strong nucleophile to attack adefovir phosphonoimidazolide, but carboxylate group, a traditional functional group which can be easily activated by EDC/sulfo-NHS. The use of this linker facilitates the activated form of adefovir’s derivative (i.e. sulfo-NHS ester of adefovir phosphonoacetate) to be attacked by primary amines from hemoglobin surface, allowing the conjugation to be performed at neutral pH and with shorter stirring time, and significantly reducing the risk of protein’s deamidation and aggregation. The application of this phosphonoacetate linker can be extended to the entire groups of acyclic nucleoside phosphonates (e.g., tenofovir and cidofovir) [40, 151] and cyclic nucleoside phosphonates.

At last, a new ICP-MS based method was developed to detect and quantitate exogenous Hb·Hp complex, a promising carrier for adefovir, by substituting iron-bound heme group with the gallium(Ga)-bound protoporphyrin IX in Hb prior to complexing it with Hp. While Ga-labeled free Hb appears to be only marginally stable, the Hp/Hb$_{\text{Ga}}$ complex displays remarkable stability on a time scale relevant for pharmacokinetic measurements. Gallium is a non-essential metal and is not present in humans (absent any environmental or workplace exposure to Ga-containing materials), which makes it a convenient tag that is suitable for detection and quantitation of exogenous Hp/Hb$_{\text{Ga}}$
complexes in biological matrices using ICP MS as a method of detection. Despite several spectral interferences, Ga can be detected with sensitivity that is more than adequate for pharmacokinetic studies of Hp-based therapeutics.

5.2. Future direction

The future directions of this dissertation include: (1) evaluation of the chemical stability of protein/adeovir conjugates; and (2) investigation of pharmacokinetic (PK) /pharmacodynamics (PD) profiles of protein conjugated adefovir payloads.

Chemical stability of the linkage between protein and its payload during the transport and after arrival at therapeutic targets is an important property of the protein/drug conjugates, which needs to be evaluated with regards to the safety and efficacy. Drug cleavages which occur in the serum expose healthy tissues to those toxic compounds, thereby raising safety concerns. On the other hand, ineffective drug release in lysosomal environment probably leads to delayed efficacy. In order to assess the Lz/PMEA conjugates (phosphate linker)’ drug release during and after the delivery, the hydrolytic stability assay of those conjugates will be performed in mouse serum and lysosomal extract respectively [152]. Cleaved PMEA or PMEAp compounds in biological matrices will be enriched using solid phase extraction method (anion exchange resin) prior to quantitation by LC/MS/MS approach. The phosphate linkage in Lz/PMEA conjugates is predicted to have high stability in mouse serum due to the low abundance of acid phosphatase and physiological pH which is unfavorable to catalytic activities of this enzyme. Its dimerized form, a pyrophosphate linker used in antibody drug conjugates, has been demonstrated to be stable after seven-day incubation with human serum [152],
providing an adequate time window for protein mediated drug delivery. If Lz/PMEA conjugates undergoes unexpected drug release in serum, the phosphate linker can be replaced by phosphonoacetate and the resultant phosphonoester bond displays slow cleavage when adefovir phosphonoacetate compounds are incubated with low concentration of acid phosphatase in pH 5 (see Figure Appendix B). Lz/PMEA conjugates are expected to release their payloads in lysosomal extract due to the the presence of highly abundant acid phosphatase and the acidic environment which favors the catalytic activities of this enzyme. If cleavages of PMEA in lysosomal extract are not effective, it is feasible to introduce an additional phosphate between drug payloads (adefovir monophosphate) and protein carrier to provide more space for acid phosphatase’s binding and to facilitate the drug release [152]. Therefore, the optimized protein/PMEA conjugates should maintain high stability in serum or other extracellular environment, but be vulnerable to cleavages in the lysosome.

In addition to chemical stability of the linkage between adefovir and its protein carrier, pharmacokinetic(PK)/pharmacodynamics(PD) profiles of the protein conjugated adefovir are also very important. The concentration of these linked drug payloads can be calculated using their protein carriers’ concentration in basis of fixed drug to protein ratio (DPR). Therefore, in this dissertation, we developed a novel strategy to quantitate adefovir’s protein carriers, Hb-Hp complex, by using gallium as a tracer and ICP-MS as an analytical technique. However, quantification of protein carriers alone is not sufficient for investigation of PK/PD properties of the conjugated drugs because the linkages between
the payloads and carriers may undergo cleavages in extracellular environment, resulting in varying DPRs which pose challenges to calculation of payloads’ concentration.

To address this problem, a p-cymene ruthenium (Ru) tag designed for protein conjugated adefovir phosphonoacetate is currently being developed in our lab. The preliminary MS data (see Figure 6.1) indicates a successful chelation of adefovir phosphonoacetate with p-cymene ruthenium and a 1:1 adefovir to Ru ratio. The use of dual metal labeling in quantitation of Hb-Hp complex/defovir conjugates will provide us absolute concentrations of Ga and Ru, as well as Ru/Ga ratio, allowing us to track both protein carriers and linked drugs in biological matrices. Since both Ru and Ga are non-covalent tags, unexpected release of those metals during the delivery may take place, thereby impacting the accuracy of this method. To evaluate the influence of metal loss on the quantitation of drugs and proteins, dual-radiolabeled Hb-Hp complex/defovir conjugates will be prepared by conjugating defovir (adenine-8-14C) phosphonoacetate with 3H labeled hemoglobin (βCys93 residues are conjugated with N-ethyl-1,2-3H-maleimide) and used to validate the metal labeling method [153]. Those radiolabels are covalently attached to the linked drugs and proteins, overcoming the adverse impacts of label loss on method’s accuracy. Dual channel liquid scintillation counter will be used to record radioactivities of the biological samples, and to convert the rediosignals to concentrations of defovir and protein carriers. If the concentrations of linked drugs and their protein carriers, which are measured by metal labeling approach (ICP-MS), are lower than those determined by radiolabeling method, p-cymene Ru and gallium protoporphyrin IX may undergo dissociation. To solve these problems, ligands (e.g., PTA,
1,3,5-tri-aza-7-phosphaadamantane) can be selected to adjust the linked drugs’ coordination to ruthenium [154], improving the stability of Ru/adeovir complex. Some metal (e.g., indium) protoporphyrin IX compounds or their derivatives can be evaluated their ability to bind with hemoglobin. If the concentrations of conjugated adefovir and protein carriers determined by both approaches are consistent, metal labels are stably attached the conjugates and this dual metal labeling method is accurate and reliable.

In addition to the accuracy and reliability of this ICP-MS based quantitation method, the selectivity of metal labeled protein conjugates during the delivery also needs to be assessed because some labels (including ligands) may alter the protein surface property. For example, the attachment of p-cymene ruthenium compounds to linked drugs might increase the lipophilicity of the entire protein/adeovir conjugates, facilitating conjugate’s cellular entry via lipid uptake pathway instead of CD163 receptor mediated internalization. This impact of p-cymene ruthenium tag on selectivity of protein conjugates in delivery can be evaluated by comparing the labeled conjugates’ cellular uptake in CD163+ and CD163- cells. If both Ru and Ga are detected by ICP-MS in the lysates of CD163- cells, the incorporation of p-cymene ruthenium with protein conjugates allows lipid uptake pathway to compete with CD163 mediated internalization during cellular uptake process. Under this circumstance, p-cymene can be replaced by other ligands with lower lipophilicity such as imidazole.

If this dual metal labeling approach is accurate and does not affect the selectivity of conjugates’ delivery, it can be used to evaluate the cellular uptake of Hb·Hp complex conjugated adefovir in macrophages. These CD163 positive cells exposed to dual metal
labeled protein/drug conjugates for varying time points will be lysed, the Ru and Ga concentrations in the lysates will be measured by ICP-MS and used to quantitate internalized linked adefovir compounds and their protein carriers respectively. Time course of intracellular concentration of conjugated drugs which gain the entry by CD163 mediated internalization will be established and compared with that of free adefovir compounds which enters cells via organic anion transporters. A higher intracellular concentration of those linked drugs will demonstrate the ability of CD163 receptors to elevate the cellular uptake of Hb·Hp complex conjugated adefovir. A lower intracellular concentration of protein linked adefovir indicates a possible saturation of CD163 receptors by Hb·Hp complex. To address this problem, DPR of the protein/drug conjugates can be elevated by elongating reaction time or using more effective synthetic strategy. Another explanation for a lower intracellular concentration of conjugated drugs is an interruption of CD163’s recognition on Hb·Hp complex by random conjugation. To solve this problem, site specific conjugation of adefovir with protein carriers can be performed by engineering cysteines (usually serine residues are replaced by cysteine residues) into regions which are irrelevant to any protein associations and modifying adefovir with maleimide to target those cysteine residues on protein surface.

Beyond investigation of PK properties, the use of ruthenium tag for protein conjugated adefovir’s quantitation can be also extended to profiling drugs’ pharmacodynamics. The intracellular Ru concentration of HIV-infected macrophages after internalization of metal labeled Hb·Hp complex/adefovir conjugates can be correlated with drugs’ suppressive activities of HIV’s reverse transcription, generating an
exponential curve which determines the half maximum inhibitory concentration (IC₅₀) of protein linked adefovir. If the IC₅₀ of the conjugated drugs is higher than that of their free counterparts which enter cells via organic anion transporters, the cleavages of phosphonoester bonds in protein conjugated adefovir phosphonoacetate maybe delayed or not completely. Under this circumstance, an extra phosphate can be introduced between adefovir and phosphonoacetate, producing a compound which is called adefovir monophosphate phosphonoacetate. This additional phosphate group provides more space for lysosomal acid phosphatase’s binding and facilitates drug release, probably decreasing IC₅₀ of protein conjugated adefovir.

In summary, chemical stability of protein/adefovir conjugates in various biological matrices (including serum and lysosomal extracts) will be evaluated in the future to support optimization of conjugation chemistry. In addition, an accurate, reliable and robust quantitation method using metal as labels and ICP-MS as analytical technique will be developed in the future to investigate PK/PD profiles of conjugated adefovir compounds.
Figure 5.1. MS/MS analysis of ruthenium labeled adefovir phosphonoacetate (m/z 630 shown in the insert). The ion peak (m/z 586) corresponds to the resultant complex which loses carboxylate group ($\Delta m= 44$ Da). The ion peak (m/z 508) represents that complex which undergoes cleavage at phosphonoester bond close to phosphonoacetate moiety ($\Delta m= 122$ Da). The ion peak (m/z 375) corresponds to the complex which undergoes cleavage at phosphonoester bond close to adefovir moiety ($\Delta m= 255$ Da).
APPENDIX A

HYDROLYTIC TEST OF ADEFOVIR PHOSPHONOIMIDAZOLIDE
Method: hydrolytic tests of adefovir phosphonoimidazolide (PMEA-Im) (diluted 1/100 from original DMF solution, final PMEA-Im’s concentration: 122 mM) were performed in 150 mM Tris-HCl buffer at pH 7.5. Hydrochloric acid was added to decrease the solution pH from 7.5 to 5.5 after 1 hour of incubation. Chromatographic analysis of different hydrolytic samples was carried out with Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatograph equipped with a 2 mm × 250 mm ProPac® SAX-10 (Dionex, Sunnyvale, CA) strong anion-exchange column. Ammonium acetate at pH 7.0 was used for gradient elution (50 mM in mobile phase A and 750 mM mobile phase B). The flow rate was maintained at 0.2 mL/min. The linear gradient was run from 0% to 100 % mobile phase B during a 20 min time period. UV absorbance at 260 nm was used for detection.

Results and discussions: adefovir (PMEA) sample (black trace) (see Figure Appendix A) was run as a control to determine the elution sequence of those analytes which carry different charges at pH 7. The peaks which elute before and after PMEA represent PMEA-Im and dimeric form of PMEA respectively. Since both PMEA and its dimeric form carry two negative charges at pH 7, their peaks highly overlap, but are distinguishable. Chromatograms (light blue and blue traces) (see Figure Appendix A) of diluted PMEA-Im’s hydrolytic samples (PMEA-Im’s concentration 1.2 mM) indicate a slow hydrolysis at pH 7.5. The addition of hydrochloric acid (red trace) accelerates PMEA-Im hydrolysis (see Figure Appendix A). The extremely high abundance of PMEA and its dimeric form in ether precipitated PMEA-Im sample (see purple trace in Figure Appendix A) suggests severe hydrolysis and dimerization. This can be explained by that those PMEA-Im compounds are highly condensed during the precipitation process (the volume was decreased from 300
μl (DMF solution) to 30-40 μl (solid)). If there is a trigger such as acid or water molecules, the rate of PMEA-Im’s hydrolysis and dimerization during precipitation process will be significantly higher than in diluted solution. The hydrolysis and dimerization of PMEA-Im during the ether precipitation, to some extent, can be defined as quenching of reaction intermediates.
Figure Appendix A. Chromatograms of hydrolytic samples of PMEA-Im.
APPENDIX B

HYDROLYTIC TEST OF ADEFOVIR PHOSPHONOACETATE
Method: hydrolytic test of adefovir phosphonoacetate (PMEApa) was performed in 150 mM NH₄Ac buffer (pH 5) at 37°C. Acid phosphatase from sweet potatoes was added to the solution at a final concentration 1.5 mg/ml, equivalent to 0.45-4.5 unit/ml. Incubated samples were filtrated those enzymes by using 10 kDa cutoff prior to injection into LC. Chromatographic analysis of hydrolytic samples at different time points (0, 24, 48 and 72 hours) was carried out with Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatograph equipped with a 2 mm × 250 mm ProPac® SAX-10 (Dionex, Sunnyvale, CA) strong anion-exchange column. Ammonium acetate at pH 7.0 was used for gradient elution (50 mM in mobile phase A and 750 mM mobile phase B). The flow rate was maintained at 0.2 mL/min. The linear gradient was run from 0% to 100 % mobile phase B during a 20 min time period. UV absorbance at 260 nm was used for detection.

Results and discussions: the starting material for this acid phosphatase mediated hydrolytic test consists of adefovir (7.2 min), dimeric form of adefovir (7.9 min) and adefovir phosphonoacetate (18.3 min). The black, red, blue and brown traces correspond to hydrolytic samples after 0, 24, 48 and 72 hours incubation (see Figure Appendix B). The half-life for the decay of PMEAp (cleavage of phosphonoester bond) can be estimated to be 72 hours based on the integration of the peaks which elute at 18.3 min. This result indicates that the phosphonoester linkage (or bond) in adefovir phosphonoacetate compounds are cleavable when incubated with low concentration of acid phosphatase. The half-life of this hydrolysis provides an adequate time window for successful delivery of adefovir phosphonoacetate liked hemoglobin to its target site,
Figure Appendix B. Chromatograms of hydrolytic samples of adefovir phosphonoacetate.
APPENDIX C

TOP-DOWN ANALYSIS OF HEMOGLOBIN ADEFOVIR CONJUGATE
Method: Hb/PMEA conjugates were purified using a Waters BioSuite™ C18 column (100Å, 3 µm, 2.1×50 mm) at a flow rate of 0.25 mL/min with the elution shown in Table 3.1. Protein conjugates fractions were collected, mixed and analyzed by top down analysis performed on a Bruker Solarix 7.0T Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA). Collisionally activated dissociation (CAD) of the mass-selected Hb (α)/(PA)2(PMEA)2 conjugate was carried out using the parameters as follows: isolation window 993±5 Da; collision energy: 15 eV.

Results and discussion: CAD of Hb (α)/(PA)2(PMEA)2 ion at +16 charge state (m/z 993.5) gives rise to a large number of fragment ions. Singly charged fragment ions are observed in the low- m/z region of the mass spectrum (m/z 256 and 274) and their complementary fragments are located at high m/z region [m/z 977.5 (+16), 1042.6 (+15) and 1041.4(+15)] (see Figure Appendix C1). The most abundant of the two low-mass fragments (m/z 274) corresponds to a (PMEA+H)+, produced by the cleavage of phosphonoester bond which is close to protein carrier. The complementary ion gives is represented in the CAD mass spectrum with an abundant peak at m/z 1041.4 (charge state +15). The other ion (m/z 256) is originated from the cleavage of phosphonoester bond which is close to adefovir. Its corresponding complementary fragments at high m/z region are m/z 977.5 (+16) and 1042.6 (+15). Ion peaks [i.e. 961.5 (+16) and 1024.4 (+15)] representing conjugates which undergo double 255 Da loss were observed during the fragmentation (see Figure Appendix C1). Besides those fragmentations which occur on protein linked PMEAp, CAD also induces cleavages of polypeptide amide bonds, providing location information of the drug conjugations (see Figure Appendix C1, C3 and C4). Although phosphonoester bonds
in protein conjugated PMEAp a are labile in the gas phase and probably undergo
dissociation prior to peptide fragmentation, the resultant residues (i.e.
phosphonoacetylated peptides or their dehydrolyzed form) (see their structures in Figure
Appendix C2) can be used to map conjugation sites of PMEAp a. The presence of Hb (α).derived peptide fragments in the CAD mass spectrum of the Hb/PMEA conjugate is not
surprising since the precursor ion represents Hb (α)/(PA)₂(PMEA)₂. What is surprising is
that the majority of the peptide fragments are derived from Hb (β). This can be explained
by that the mass isolation includes Hb (β)/(PA)₄(PMEA)₂ (m/z 993, charge state +17) which
has very close m/z value with Hb (α)/(PA)₂(PMEA)₂ (m/z 993.5, charge state +16). Analysis
of those peptide fragments indicates that N-terminal primary amine-containing residues
such as V1, K7, K11 and K16 on Hb (α) and V1, K8 and K17 on Hb (β) are probably the
conjugation sites in this reaction. Conjugation at those residues on Hb are not that
surprising because some of them (including βV1, αV1, βK8 and αK11) have been evaluated
to have very high reactivity in Hb PEGylation (Hu, T. Biochem. J., 392, 555-564 (2005)).
Figure Appendix C1. Mass spectrum of fragment ions produced by collisional activation of 1:2 Hb(α)/PMEA conjugate (m/z 993.5). The diagram shows the structure of observed fragment ions.
Figure Appendix C2. Structures and labels of PMEAp linked peptides and their CAD induced residues.

* represents the modified peptides with intact PMEAp

** represents the phosphonoacetylated peptide (phosphonoester bond close to adefovir undergoes cleavage)

*** represents the dehydroxylphosphonoacetylated peptide (phosphonoester bond close to protein undergoes cleavage)
Figure Appendix C3. Zoomed view (m/z 650-950) of mass spectrum of fragment ions produced by collisional activation of 1:2 Hb(α)/PMEA conjugate (m/z 993.5).
Figure Appendix C4. Zoomed view (m/z 1050-1400) of mass spectrum of fragment ions produced by collisional activation of 1:2 Hb(α)/PMEA conjugate (m/z 993.5).
Figure Appendix C5. CAD fragmentation map derived from data shown in Figure Appendix C1, C3 and C4.
APPENDIX D

PREPARATION OF P-CYMENE RUTHENIUM LABELED ADEFOVIR PHOSPHONOACETATE
Method. Preparation of ruthenium labeled adefovir phosphonoacetate is shown in Figure of Summary and Future Direction. 11 mg dichloro(p-cymene)ruthenium(II) dimer (0.018 mmol) was dissolved in acetone (dark brown), followed by addition of 18.8 mg silver trifluoromethanesulfonate (0.072 mmol). After 30 min stirring, light yellow precipitates (i.e. silver chloride) was centrifuged down and the supernatant (orange) was placed in another glass vial to interact with 14.4 mg adefovir phosphonoacetate (0.018 mmol). After 4-hour incubation (stirring), brown precipitates were collected and dissolved in water for MS analysis.
APPENDIX E

DETERMINATION OF LIMIT OF DETECTION AND LIMIT OF QUANTITATION IN QUANTITATION OF GA LABELED HEMOGLOBIN HAPTOGLOBIN COMPLEX USING ICP-MS
Method: Quantitation of gallium in serum samples spiked with Hp-HbGa was carried out using NexION 300X (PerkinElmer, Waltham, MA) ICP mass spectrometer. The kinetic energy discrimination (KED) mode was used to reduce polyatomic interference. Gallium quantitation in serum was based on an external calibration curve obtained with a standard solutions of Ga (0.01 to 1 μg L⁻¹), which also contained Rh (1 μg L⁻¹) as the internal standard. Intensities of ionic signals at m/z 69 (⁶⁹Ga), 71 (⁷¹Ga) and 103 (¹⁰³Rh) were used to calculate the ⁶⁹Ga/¹⁰³Rh and ⁷¹Ga/¹⁰³Rh ratios, from which calibration curves were constructed. Rh was also added to all serum samples (to a final concentration of 1 μg L⁻¹) following their overnight digestion at 37 °C in a mixture of nitric acid (49%) and hydrogen peroxide (7%). The limit of detection (LOD) of this method was calculated using equation as follows:

\[ \text{LOD}_x = \frac{3 \times \text{slope}}{\sqrt{\frac{\sum (y_i - \bar{y})^2}{n-2}}} \]

\( y_i \) is measured value; darkened \( y_i \) is fitted value on the calibration curve.
Figure Appendix E. Evaluation of the feasibility of quantitation based on Ga isotopes based on external calibration curves obtained with Rh as the internal standard. Intensities of ionic signals at m/z 69 (\(^{69}\text{Ga}\)), 71 (\(^{71}\text{Ga}\)) and 103 (\(^{103}\text{Rh}\)) are used to calculate the \(\frac{^{69}\text{Ga}}{^{103}\text{Rh}}\) and \(\frac{^{71}\text{Ga}}{^{103}\text{Rh}}\) ratios, from which calibration curves are constructed. The two calibration curves (\(\frac{^{69}\text{Ga}}{^{103}\text{Rh}}\) and \(\frac{^{71}\text{Ga}}{^{103}\text{Rh}}\)) have linear regression coefficients \(r^2\) of 0.995 and 0.994, respectively. The limit of detection (LOD) for Ga is calculated to be 0.03 ppb, the limit of quantitation is calculated to be 0.1 ppb using the commonly accepted algorithms (Miller, J.N., Miller, J.C. Statistics and Chemometrics for Analytical Chemistry, 6th edition. Pearson Education Limited, Essex, UK, 2010). These LOD and LOQ numbers correspond to \(\text{Hb}_{\text{Ga}}\) concentration levels 0.12 nM and 0.41 nM. Assuming the total labeling ratio is 3.5:1.
APPENDIX F

CATION EXCHANGE CHROMATOGRAM OF LYSOZYME ADEFOVIR CONJUGATE
**Method:** IXC (cation exchange) was performed on Agilent 1100 (Agilent, Santa Clara, CA) liquid chromatograph equipped with a 2.1 mm × 100 mm PolyCAT A (PolyLC Inc., Columbia, MD) weak cation-exchange column (particle size of 5 μm and pore size of 300 Å). Ammonium acetate at pH 7.0 was used for MS-compatible gradient elution (100 mM in mobile phase A and 1 M mobile phase B). The flow rate was maintained at 0.1 mL/min, and the linear gradient ran from 0% to 60% mobile phase B during a 60 min time interval.
Figure Appendix F. UV chromatogram (cation exchange) of Lz/PMEA conjugate produced from pH 6.8.


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