March 2024

High Resolution Mass Spectrometry as a Platform for the Analysis of Polyoxometalates, their Solution Phase Dynamics, and their Biological Interactions.

Daniel T. Favre
University of Massachusetts Amherst

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High Resolution Mass Spectrometry as a Platform for the Analysis of Polyoxometalates, their Solution Phase Dynamics, and their Biological Interactions.

A dissertation presented

by

DANIEL T. FAVRE

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

DOCTOR OF PHILOSOPHY

February 2024

Chemistry
High Resolution Mass Spectrometry as a Platform for the Analysis of Polyoxometalates, their Solution Phase Dynamics, and their Biological Interactions.

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Department of Chemistry
DEDICATION

To my friends and family,
for being there when I needed them.
ACKNOWLEDGMENTS

I want to thank my advisor Igor Kaltashov for his guidance and support throughout my graduate career. I would also like to thank my committee members, Richard Vachet, Kevin Kittilstved, and Stephen Eyles for their help and guidance throughout this project. I would like to thank Debbie Crans and her lab at Colorado State University and Matthew Miller and his lab at McMaster University for their contributions to the decavanadate and SARS-CoV-2 research respectively.

Thanks to the NIH (R01 GM112666) and the NSF (CHE-1709552) for their financial support and to the Mass Spectrometry Core facility at UMass-Amherst (RRID:SCR_019063) for providing and maintaining the mass spectrometry instrumentation used in this work. A special thanks to all my fellow Kaltashov lab members for their assistance throughout this work as well as to my friends and family for their support throughout all stages of my education.
ABSTRACT

High Resolution Mass Spectrometry as a Platform for the Analysis of Polyoxometalates, their Solution Phase Dynamics, and their Biological Interactions.

February 2024

Daniel Favre, B.A., College of the Holy Cross
Ph.D., University of Massachusetts Amherst
Directed by: Professor Igor Kaltashov

Polyoxometalates (POMs) are a class of inorganic molecule of increasing interest to the inorganic, bioinorganic and catalytic communities among many others. While their prevalence in research has increased, tools and methodologies for the analysis of their fundamental characteristics still need further development. Decavanadate (V10) specifically has been postulated to have several unique properties that have not been confirmed independently. Mass spectrometry (MS) and its ability to determine the composition of solution phase species by both mass and charge is uniquely well suited to the analysis of POMs. In this work we utilized high-resolution mass spectrometry to characterize V10 in aqueous solution. We were able to observe a unique loss of water fragmentation pathway and prove the existence of partially reduced V10 species in solution. Due to the high negative charge of the V10 complex, it was also investigated as an analogue to heparin sulfate which has been shown to disrupt the infectivity cycle of SARS-CoV-2. Native mass spectrometry was used to demonstrate the ability of V10 to bind to the receptor binding domain of the spike protein. Additionally, the ability to disrupt the formation of a complex between the receptor binding domain (RBD) of the spike protein and the ectodomain of the angiotensin-converting enzyme 2 (ACE2) receptor was likewise
investigated using native MS. This mechanism of action against SARS-CoV-2 was confirmed with cell viability studies. Further investigation of the solution phase dynamics of V10 was carried out by performing an oxygen exchange experiment coupled with high resolution mass spectrometry. The resulting exchange profiles revealed the existence of multiple populations of V10, including novel metastable conformations of V10. Further analysis of the oxygen exchange data yielded kinetic information about these metastable states and their role in the solution phase dynamics of V10. Finally, we also demonstrated the utility of bulk electrolysis in the synthesis of POV nanocages with unique ratios of reduced V\textsuperscript{IV} centers. MS analysis of the species showed that the VO\textsubscript{4} centered nanocage V\textsubscript{19}O\textsubscript{46} is a precursor to the more stable chloride centered V\textsubscript{15}O\textsubscript{36}Cl which was shown to have unique solution phase dynamics upon subsequent oxygen exchange analysis. The work presented demonstrates the utility of mass spectrometry and isotopic exchange in the analysis of POMs.
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INTRODUCTION

Polyoxometalates (POMs) are a subclass of inorganic complexes consisting of multiple metal oxide subunits assembled into a larger structure. A unique aspect of POMs is that they often self-assemble in solution under the appropriate conditions making their synthesis comparatively simple in many cases. POMs are of increasing interest to many disciplines as they occupy a niche between bulk oxometallate solids and single-molecule species. One field they are particularly prevalent in is catalysis in which POMs are considered attractive catalysts candidates due to the ease with which many parameters (including redox activity) can be tuned. Additionally, the ease with which POMs can be synthesized and the capability as an electron acceptor make them particularly appealing as a catalyst for green fuel production. The ability to act as an electron acceptor (and to do so multiple times) also makes POMs attractive to the field of electronics, in which these properties make POMs potential charge-storages units in a theoretical memory cell. Their charge storage capabilities alongside the thermal stability and low cost of synthesis of POM films make POMs an increasingly attractive choice in the field of electronics.

POMs have also become increasingly prevalent in biomedical research, again in part due to the range of potential structures and compositions available. As a potential antibiotic POM research has shown that they can be active against antibiotic resistant bacterial strains, making them a compelling complementary treatment option alongside traditional antibiotics. POMs have also been shown to function as antivirals by disrupting the binding of viral particles to host cells, disrupting the viral particle itself, and inhibiting viral replication. Antitumor properties of POMs have also been observed via apoptosis and other pathways. Other reported mechanisms of action against cancer cells include DNA binding, enzyme inhibition, and binding to HSA.
The POM Decavanadate (V10) in particular has been a focus of the research community being a stable POM comprised of 10 vanadium centers and 28 oxygens.\textsuperscript{14} In the field of biomedicine V10 has been shown to interact with a variety of proteins such as inhibition of myosin, or inhibition of enzymes like ATPases.\textsuperscript{15-16} V10 compounds have also been shown target cancers by limiting their growth or through apoptosis.\textsuperscript{17-18} Antibacterial effects of V10 have been reported as well, showing a significant impact on bacterial growth.\textsuperscript{19-20} Like other POMs V10 has also been shown to have catalytic activity including photocatalysis based oxygenation of sulfides, and catalysis of azide alkyne cycloaddition reactions.\textsuperscript{21-22} Worth noting is the ability to modulate the activity of V10 for many of the listed applications by modifying the V10 structure with organic function groups, or substituting one or more of the vanadium metal centers with alternate transition metal atoms.\textsuperscript{17-20}

One of the most common techniques used in the analysis of POMs has been x-ray crystallography, which is capable of determining the structure of inorganic complexes along with counterions and measuring bond angles and lengths.\textsuperscript{23} Despite this it has difficulty capturing the heterogeneity of POMs in solution, particularly for less abundant or short lived species. Another commonly used technique is NMR, which can differentiate coexisting POM species and identify structurally unique metal or oxygen centers (when spin active nuclei for those atoms exist).\textsuperscript{24-25} NMR also enables isotopic exchange studies by observing the exchange to or from spin active nuclei which can describe the kinetics/lability of those atoms in the POM complex.\textsuperscript{26} NMR lacks the ability to tie the state of a particular signal to the state of the whole molecule, and in the case of oxygen exchange makes it unable to determine whether exchange in one region of the molecule coincides with exchange in other regions. Mass spectrometry has previously been applied to the analysis of POMs, commonly to differentiate species in solution and confirm their identity.\textsuperscript{27-28} Many studies lack the resolution and mass accuracy needed to accurately identify POM species,
in particular they do not leverage the ability of MS data to determine the composition of oxidation states observed. MS has also been used to perform isotopic exchange studies like those done with NMR, but it either resulted in an average exchange level across all POMs in solution or was done under conditions that limited the information that could be gleaned from it.\textsuperscript{29-30}

Due to the increasing size, complexity, and number of POMs used in research, the need for a more robust suite of analytical tools for their analysis grows.\textsuperscript{2} Mass spectrometry and its related techniques (ion mobility, LC-MS) offer an avenue for the analysis of POMs that can yield a better understanding of their composition than modeling. Not only does it allow for the analysis/isolation of specific isotopic composition, but it can also differentiate POMs by charge state which would describe the distribution of oxidation states of their constituent metal centers. Ion mobility would not yield as detailed structural information as crystallography, but the ability to tie structural differences to specific isotopic compositions or oxidation states makes it a notable complementary technique. MS allows for the possibility of confirming POM characteristics that were postulated or previously predicted computationally.\textsuperscript{31-32} Researchers investigating the anticancer properties of POMs have postulated that some of the therapeutic effects may originate from smaller species or other unidentified complexes which could be identified by MS analysis.\textsuperscript{12} The presence of coexisting POM species and the conversion to other conformations also impacts catalytic applications wherein these different species may have differing catalytic activity.\textsuperscript{3} These other species and they rate of their emergence could be determine through a combination of high resolution mass spectrometry and isotopic exchange analysis.

Many transition metals have complex isotopic distributions, which is further complicated by the higher quantity of metal centers present in POMs. Furthermore, transition metals can occupy multiple oxidation states which can cause heterogeneity in the overall charge of the POM structure. These variables make accurate and complete speciation of POMs difficult, especially
when conducting time sensitive experiments or working with oxygen sensitive or otherwise unstable species. The multiple oxidation states of POMs and the ability to transition between them play a particularly important role in catalytic use of POMs, in which almost all uses involve reduction of one or more POM metal centers. The heterogeneity of POM oxidation states is currently a significant limiting factor in their catalytic uses and the ability of MS to differentiate between unique compositions of oxidation states would help in identifying potential contaminants in synthesized catalysts.

The increasing biomedical applications of POMs, including antibacterial, antiviral, and anticancer activity among others, also calls for a more robust speciation of potential drug-like molecules. Determining the mechanism of action of this activity is an important part of developing these inorganic drug-like molecules. Native mass spectrometry and its ability to identify protein complexes and adducts makes it well suited to studying POMs ability to bind to and disrupt their activity. The ability of MS to differentiate POM species is also important to their biomedical applications as different POM species are believed to have significantly different levels of cytotoxicity, a significant limiting factor for this application.

High resolution mass spectrometry also makes it possible to analyze POMs using isotopic exchange techniques in a similar manner to how hydrogen deuterium exchange is used in the study of proteins and their dynamics in solution. Isotopic exchange techniques make it possible to observe transient dynamic events that would otherwise be difficult to observe. The unique conformations being sampled could impact potential uses of POMs, changing its binding affinity, and catalytic activity, among other properties. These dynamic events could also be particularly important in determining the stability of POV nanocages, a class of oxovanadate based POMs known for their ability to package other molecules. Dynamic events that expose the payload of
these nanocages would diminish the ability of the nanocage to retain or protect its payload (possibly by preferentially being reduced in place of the payload).\textsuperscript{34}

Chapter I of this work focuses on the analysis of V10 using high resolution mass spectrometry (HRMS) and establishes the ability of MS analysis to differentiate POMs with unique compositions of oxidation states. It also shows how POM stability and factors impacting it can be studied using ms/ms analysis. These techniques are relevant to the future studies of POM catalysts as POM contaminants and the stability of the catalyst can both impact functionality.\textsuperscript{3} Correct identification of species is also important in biomedical applications in which POMs are often employed under conditions where they are not considered stable.\textsuperscript{7} In most potential applications of POMs the ability to accurately identify the POM of interest and confirm its stability is vital to identify the role the POM plays in that system.

In chapter II of this work native mass spectrometry was employed alongside HRMS to determine the mechanism of action by which V10 could disrupt the SARS-CoV-2 infectivity cycle. The native MS data demonstrates the ability to observe POMs binding to proteins and even localize that binding to specific regions using a model peptide. This is particularly relevant to work wherein POMs are used as anticancer agents wherein the mechanism of action is not well understood. For these applications confirming the mechanism of action would allow for better selection of POM candidates tailored to the target identified.\textsuperscript{12}

Chapter III of this work demonstrates the utility of oxygen exchange as a tool for the analysis of POM dynamics in solution when coupled with HRMS. The metastable states that can be observed using this technique can further the POM community’s understanding of their solution phase dynamics and inform their potential use cases. Transiently sampled POM configurations could impact their catalytic activity, stability in electronic devices, and biological
interactions.\textsuperscript{3, 5, 12} Additionally the kinetic information gained from oxygen exchange analysis would help in controlling the emergence of these species by tuning their environment.

The final main section of work presented in chapter IV focuses on the development of a novel bulk electrolysis synthesis of POV nanocage species. It also utilizes the toolkit developed in the work shown in the previous chapters to characterize the POV species synthesized and compare them to previously synthesized species. Studying these POV nanocage species is a practical demonstration of how a newly synthesized POM, or a POM of interest, can be robustly characterized using a MS toolkit. Even low abundance species present in solution could be identified by empirical formula including the distribution of oxidations states. Dynamics and transiently sampled conformations can be probed using oxygen exchange studies coupled with HRMS. In the following work we demonstrate the utility of MS for the analysis of POMs, propose and validate and oxygen exchange method for the study of POMs, and develop a novel synthetic pathway for POV nanocages.
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[X@HV(IV) 8 V(V) 14 O54 ](6-) self-assemblies with symmetry-breaking guest anions. Chemistry 2015, 21 (6), 2387-97.
A: Introduction

Polyoxometalates (POMs) are a unique class of polynuclear inorganic compounds, which are mostly comprised of transition metal and oxygen atoms, although other atoms (such as phosphorus and silicon) may also be involved. POMs are frequently viewed as the “missing link” between the single-molecule and the bulk material scales. Apart from their obvious importance for the field of mesoscopics and helping understand how the physical properties of materials are determined by the chemical properties of constituent molecules, POMs continue to enjoy considerable attention in fields as diverse as catalysis, electronics and medicine. Decavanadate (V$_{10}$O$_{28}^{6-}$, or V10) is one particularly interesting member of the POM family, which had been shown to interact with a variety of biomolecules in vivo, eliciting a range of biological effects that are distinct from those typically associated with smaller oxovanadate species. Despite being a focus of extensive research efforts in the past two decades, several aspects of V10 structural and chemical properties remain incompletely understood. As is the case with many other POMs, V10 can interconvert between multiple species in solution, but the mechanisms of such processes (which likely include an intricate interplay of acid-base and redox chemistry) remain elusive. For example, the existence of a partially reduced V10 species (V$_{10}$O$_{26}^{4-}$) has been reported based on spectroscopic measurements, but never confirmed independently. Likewise, the proton association with V10 species in solution and the hydrogen bonding phenomena have been shown to play important
roles in dissociation/association of this paradigmatic member of the POM family.\textsuperscript{11} Lastly, the notion of the low-energy metastable configurations of V10 (distinct from its crystal structure\textsuperscript{12}) has been invoked to explain several phenomena related to its interaction with the solvent.\textsuperscript{13,14}

The majority of work on V10 (as well as other POMs) characterization has been traditionally carried out using X-ray crystallography in the solid state and spectroscopic methods in solution. While the former provides atomic-level structural data for stable POM molecules, it is insensitive to those species that are present in solution in minute quantities or become populated only transiently. In contrast, NMR can be used to speciate heterogeneous populations of POMs. The natural abundance of the \textsuperscript{17}O isotope and the NMR activity of many metal centers allow for facile application of the technique to a wide array of POMs. However, the atomic-level resolution afforded by the NMR is frequently insufficient for complete characterization of distinct ensembles of POMs that may co-exist in solution. Indeed, while the state of a single atom and its unique chemical environment can be monitored with unprecedented precision using NMR, grouping such states together to yield distinct molecular states is not straightforward.

In comparison to X-ray crystallography and NMR, mass spectrometry (MS) has played a less prominent role in characterization of POMs, even though the initial application of MS in this field dates back nearly four decades ago.\textsuperscript{15} Introduction of electrospray ionization (ESI) and, to a lesser extent, matrix-assisted laser desorption/ionization (MALDI) provided further impetus to developing robust MS-based methods to characterize a variety of POMs,\textsuperscript{16-19} including polyoxovanadates.\textsuperscript{20} In many ways, MS offers an attractive alternative for the analysis of POMs compared to the traditional approaches, as it enables POMs speciation in solution and allows oxidation states to be distinguished from one another. At the same time, the possibility of ion fragmentation occurring in the gas phase raises the concern of introducing artifacts in MS-based POM speciation.
measurements. Likewise, the frequent occurrence of electrochemical processes within the liquids being electrosprayed\textsuperscript{21} may cast doubt on the validity of oxidation state profiling obtained with ESI MS. In fact, the complete elimination of interfering redox processes may require the use of complicated setups and extensive front-end modification of commercial MS equipment.\textsuperscript{22} The main goal of this work was to evaluate and illustrate the utility of high-resolution MS for characterization of both structure and chemistry of decavanadate species in aqueous solutions with a particular emphasis on exploring both similarities and differences between V10 behavior in the bulk solution and in the solvent-free environment.

In addition to its obvious importance via-a-vis validation of the ESI MS-based methods of POM structural analysis, the juxtaposition of the solution- and gas-phase behavior for this class of analytes may provide important insights into the POMs/solvent interactions (and, by extension, the phenomena occurring at the insoluble metal oxide/water interface). For example, previous computational studies have indicated that the presence of hydrogen at an oxometallate surface facilitates the abstraction of oxygen from the surface.\textsuperscript{23} Oxygen abstraction from polyoxovanadate clusters has also been shown to be triggered by partial reduction of the latter,\textsuperscript{24} although no distinct partially reduced species could be detected; instead, their existence was inferred from the spectroscopic data. MS is unique in that it allows such species to be detected even if they account only for a minor fraction of the entire ensemble of molecules present in solution. In this work we use high resolution MS as an on-line detection tool for ion exclusion chromatography to provide conclusive evidence that multiple partially reduced V10 species exist in solution alongside the canonical (fully oxidized) V10 prior to the MS measurement. These reduced species accommodate a larger number of protons during the ESI process, leading to formation of ions that readily dissociate via a facile loss of water molecules and, to a lesser extent, OH\textsuperscript{-} radicals, thus mirroring processes that were previously hypothesized to take place in solution.
B: Methods

The decavanadate samples in the form of (NH₄)₆V₁₀O₂₈, Na₆V₁₀O₂₈ and K₄Na₂V₁₀O₂₈ salts were prepared using a procedure described elsewhere²⁵,²⁶ and were speciated using ⁵¹V NMR and absorbance spectroscopy.²⁷ Hydrogen peroxide (H₂O₂) and heavy water (²H₂O) were purchased from Millipore-Sigma (St. Louis, MO). All other solvents and buffers used in this work were of analytical grade or higher.

All MS measurements were carried out using a SolariX 7 (Bruker Daltonics, Billerica, MA) hybrid quadrupole-Fourier transform ion cyclotron resonance mass spectrometer equipped with a standard electrospray ionization source. V₁₀ samples were dissolved in a 5 mM aqueous solution of ammonium acetate whose pH was adjusted with formic acid to 4.85±0.15. Final decavanadate concentrations were 1 mM (negative ion mode) or 0.1 mM (positive ion mode). Decavanadate was typically kept in solution for 30 min prior to MS analysis. The ESI source conditions and ESI MS interface ion optics parameters were optimized to produce abundant signal in the 400-1100 m/z; the typical settings in the negative ion mode were as follows: ESI capillary, 3800 V; end plate offset, 500 V; nebulizer, 1.6 bar; dry gas, 4.0 L/min; dry temperature, 150 °C; capillary exit, -180 V; deflector plate, -180 V; funnel 1, -150 V, skimmer 1, -10 V; funnel RF amplitude, 200 Vₚ₋ₚ). External calibration was used to determine ionic masses in mass spectra acquired in the broad-band mode for both positive and negative ions. Post-calibration mass accuracy was determined to be at least ±3 ppm throughout the entire 200-1200 m/z region. Internal calibration was used for mass measurements in the narrow-band mode, providing mass accuracy of at least 0.3 ppm. Theoretical isotopic distributions of ionic species and accurate masses of relevant isotopologues were calculated using a web-based tool at https://www.sisweb.com/mstools/isotope.htm. Fragment ion spectra were obtained by mass-selecting precursor ions of interest in the front-end quadrupole (mass selection
window 3.0 m/z units unless specified otherwise) followed by their activation in the collision cell using argon as a collision gas. Ion exclusion LC and LC-MS measurements were carried out with an Agilent 1100 (Agilent Technologies, Santa Clara, CA) HPLC system using a 4.1 mm x 250 mm PRP-X300 (Hamilton Co., Reno, NV) ion exclusion column. The mobile phase consisted of 30 mM triethylamine, 100 mM ammonium acetate and 0.8% formic Acid. The UV detection of V10 was based on the absorbance at 254 nm. On-line LC-MS measurements were carried out by directing the eluate flow off the column directly to the ESI source of the mass spectrometer without splitting.

C: Results and Discussion

Figure 1.1. ESI mass spectrum of a 1 mM aqueous solution of sodium V10 in 5 mM ammonium acetate (pH adjusted to 4.7). The inset shows the isotopic distribution of the most abundant mono-anionic species (black trace) and the calculated isotopic distributions for the fully oxidized species ($\text{H}_5\text{V}_{10}\text{O}_{28}^-$, red bars) and a partially reduced one ($\text{H}_6\text{V}_{10}\text{O}_{28}^-$, blue).
V10 (in a variety of protonation states) is the predominant vanadium species in moderately concentrated (> 1 mM) and mildly acidic (3 ≤ pH ≤ 6) aqueous solutions, but it readily converts to other oxo-forms (e.g., VO$_4^{3-}$/HVO$_4^{2-}$ at basic pH, V$_4$O$_{12}^{4-}$/H$_2$VO$_4^{-}$ at neutral pH, or VO$_5$/HVO$_3$ upon dilution).$^{28,29}$ Although these and other smaller vanadates are expected to be present in lower quantities at mildly acidic pH as well, the negative ion mass spectrum of Na$_6$V$_{10}$O$_{28}$ acquired at pH 4.7 (Figure 1.1) shows only two oxovanadate species in the low-m/z region of the mass spectrum, V$_6$O$_{16}^{2-}$ and (H/Na)V$_4$O$_{11}^{-}$. Outside of this region, the mass spectrum is dominated by two clusters of abundant ions with distinct charge states: a group of singly charged ions (m/z region 950-1100) and the doubly charged ones (m/z 450-550). The nominal masses of the most abundant peaks in each cluster indicate that these two species (H$_7$V$_{10}$O$_{28}^{-}$ and H$_4$V$_{10}$O$_{28}^{2-}$) are produced by partial balancing of the net charge of the presumed most abundant V10 species in solution (V$_{10}$O$_{28}^{6-}$, HV$_{10}$O$_{28}^{5-}$ and H$_2$V$_{10}$O$_{28}^{4-}$) with “extra” protons or ubiquitous alkali metal cations upon electrospraying. This behavior is similar to the ESI production of the moderately-charged species representing high charge-density organic polyelectrolytes, such as heparin oligomers.$^{30}$

It is now commonly accepted that the extent of protonation (and, more broadly, multiple charging in general) of polyprotic acids in ESI MS does not reflect the acid-base equilibria in solution, but is instead dictated by the physical size of the analyte.$^{31}$ Consistent with this notion, V10 generates abundant ionic signal in the positive-ion ESI MS as well, where it is represented by H$_{17-2n-3k}$Na$_n$K$_k$V$_{10}$O$_{28}^{+}$ (n, k = 0 - 4) ions (Figure 1.2).

While the extent of protonation of the dominant V10 ionic species in ESI MS cannot be related to the acid/base equilibria in solution, the total charge accommodated by these ions in both negative- and positive ion modes is consistent with the notion of all metal ions being fully oxidized (i.e., charge state +5). Indeed, the most abundant peak in the isotopic distribution of the
mono-anionic V10 species m/z region 961-966 can be confidently assigned as the most abundant isotopologue of H5V10O28− (the calculated mass for 1H551V1016O28− is 962.3369, a value that is within 0.2 ppm of the measured mass). The lowest-mass detectable monoisotopic peak of the H5V10O28− species (m/z 961.3373) can also be readily assigned as 1H550V51V916O28− (calculated mass 961.34031, and the intensity ratio of these two isotopologues (which we will term M and M-1, respectively) is in excellent agreement with the known 51V : 50V natural abundance ratio (the theoretical isotopic distribution of the H5V10O28− is represented with red bars in the inset in Figure 1.1). However, the abundance of the third isotopologue in this distribution (M+1, measured m/z 963.3443) appears anomalously high, exceeding the expected contribution of 1H551V1017O16O27− by more than an order of magnitude. Furthermore, the spacing between the M+1 and M species (1.0071) shows a significant deviation from the mass difference between the 16O and 17O isotopes.
(1.0042) but is reasonably close to the hydrogen atom mass (1.0078). Therefore, this species was
tentatively assigned as $^1\text{H}_6\text{V}^{5+}\text{V}^{5+}\text{V}^{4+}\text{O}_{28}^-$, which represents a partially reduced decavanadate,
where an extra proton is needed to keep the overall single net charge of the anion (compensating
for the lost positive charge on one of the metals). Such satellite species are observed for all other
reasonably abundant anions containing the decavanadium core, and their mass shifts in $^2\text{H}_2\text{O}
$solutions (vide infra) confirm the presence of an “extra” hydrogen atom, lending strong support to
the notion of partial V10 reduction.

It is worth noting that the partially reduced V10 species (see the inset in Figure 1.1) are
present in all V10 samples examined in this work, regardless of the nature of the counterion (Figure
1.3). The relative abundance of the partially reduced species shows some variations among
different V10 samples with distinct counter-ions, and even within the same sample temporal variations in the intensity ratios of the $M$ and $M+1$ species are apparent (data not shown). In many instances, low-intensity ionic signal was also observed for V10 species where more than a single reduction event takes place (e.g., $H_2V^{(V)}_8V^{(IV)}_2O_{28}^-$ and $H_8V^{(V)}_7V^{(IV)}_3O_{28}^-$ incorporating two and three vanadium atoms in oxidation state IV, respectively, to ensure the charge balance). Such seemingly random variations naturally lead to a suggestion that the partial reduction occurs during the ESI process, a phenomenon that has been documented for a range of redox-active species in the past.\textsuperscript{32} Furthermore, the observation of the partially reduced V10 species in the mass spectra of V10 acquired in the presence of hydrogen peroxide in solution appears to lend further support to the notion of the ESI-driven redox processes being responsible for the partial reduction of these POM assemblies. Should the partial reduction take place in the ESI source, it would be reversed by simply switching the polarity of the ion source. However, examination of the V10 mass spectra acquired in the positive-ion mode also reveals the presence of the partially reduced V10 species, such as NaH$_7$V$_{10}$O$_{28}^+$ (Figure 1.2), contradicting the notion of V10 partial reduction being an ESI artifact.

To obtain definitive evidence with regards to the origin of the partially reduced V10 species, ion exclusion chromatography (IEC) with on-line ESI MS detection was used. IEC is a mixed-mode chromatographic modality, in which differential exclusion of weak electrolytes from the anionic resin pores is used as a physical principle enabling analyte separation.\textsuperscript{33} Although in the past IEC has been applied almost exclusively to separation of weak organic acids (based on their sizes and pK$\alpha$ values), it can be used for separation of inorganic anions as well.\textsuperscript{35,36} Since V10, as well as many smaller vanadates, are weak acids, we hypothesized that it may be possible to achieve their retention on IEC. Furthermore, partial reduction of V10 in solution should have an effect on the pK$\alpha$ values, and unless the redox equilibria are too fast on the chromatographic time scale
19

\[
e.g., \text{ the } H_3V^{(V)}_{10}O_{28}^- \rightleftharpoons H_6V^{(IV)\text{V}}_{9}O_{28}^- \rightleftharpoons H_7^{\text{IV}V_2^{\text{V}}V_8O_{28}^- \text{ interconversion occurs within } < 1 \text{ min}),}
\]

it should be possible to achieve chromatographic separation of V10 species at different oxidation states. In contrast, if the partial reduction of V10 is triggered by electrochemical processes in the ESI source, no separation of the V10 species at different oxidation states would be possible with IEC. Unfortunately, achieving efficient retention of V10 with IEC is not straightforward, as the principal species for the canonical, fully oxidized form of this POM in the pH range of interest are \(H_2V_{10}O_{28}^{4-}\) and \(HV_{10}O_{28}^{5-}\), while the pKa value of the fully protonated (electrically neutral) V10 species is significantly below 1 (and possibly below 0).\textsuperscript{37} This means that V10 retention on anionic

**Figure 1.4.** Extracted ion chromatograms representing the fully oxidized V10 (gray) and the partially reduced species incorporating one (red), two (blue) and three (olive) metal atoms at oxidation state IV obtained using ion exclusion LC with on-line MS detection. The inset shows the evolution of the ionic signal within the m/z window 961-967 as a function of elution time.
resins is very ineffective, unless an appropriate ion pairing reagent is identified. Indeed, our initial attempts to achieve V10 retention with an IEC column failed until trimethylamine (TEA) was selected as an ion pairing agent. The elution time of V10 off the IEC column in the presence of 30 mM TEA was equivalent to application of 3 column volumes of the eluent.

Importantly, the extracted ion chromatograms (XICs) recorded for the individual monoisotopic peaks representing $H_{5n}V_{10}O_{28}^-$ for $n = 0 – 3$ clearly show incongruent elution profiles (Figure 1.4). These chromatograms provide clear and conclusive evidence of the solution phase origin of the partially reduced species. Furthermore, this observation also indicates that the interconversion rate among the V10 species at different oxidation states in solution is relatively low, allowing at least partial chromatographic separation to be achieved on a time scale exceeding 10 min. Indeed, the elution profiles of chemically reactive species that are at dynamic equilibrium during the elution process are very sensitive to the kinetics of the interconversion reactions, yielding a single unresolved peak in the case of fast equilibria and allowing separation of the reactants only for systems with slower kinetics.\(^{38,39}\) The significant overlap of the elution profiles of different V10 species shown in Figure 1.4 would yield an unresolved asymmetric peak should UV absorption be used as the only means of the analyte detection. However, the unique ability of MS to distinguish the species of interest by examining the corresponding XICs allows kinetic analysis to be carried out in situations when the normal methods of detection (such as UV absorbance) fail, as was recently demonstrated for on-column association/dissociation equilibria of large protein complexes.\(^{40}\) The elution order of the four ionic species representing different oxidation states of V10 correlate with the extent of oxidation (with the fully oxidized V10 exhibiting the most efficient retention), in line with the expectation that the higher electron density (the result of partial reduction) disfavors the POM interaction with the anionic resin. Another intriguing feature revealed by the XICs shown in Figure 1.4 is the apparent correlation between the oxidation state of V10
and the peak shape. Both peak widths (measured at half-maximum) and the extent of tailing of the four XICs follow the order $\text{H}_5\text{V}^{(IV)}\text{O}_{28}^{-} >> \text{H}_6\text{V}^{(IV)}\text{V}^{(V)}\text{O}_{28}^{-} >> \text{H}_7\text{V}^{(IV)}\text{O}_{28}^{-} \approx \text{H}_8\text{V}^{(IV)}\text{V}^{(V)}\text{O}_{28}^{-}$.

The XIC shapes of reactive analytes in dynamic systems can be used to extract the rates of relevant reactions, and even though such a task is beyond the scope of this work, these calculations can be performed in the future to determine the rates of V10 partial reduction and re-oxidation.

Figure 1.5. Low-energy CAD mass spectra of $\text{H}_4\text{V}_{10}\text{O}_{28}^{-}$ (red trace), $\text{H}_5\text{V}_{10}\text{O}_{28}^{-}$ (brown) and $\text{NaH}_4\text{V}_{10}\text{O}_{28}^{-}$ (blue) acquired by placing the most abundant isotopologue of each species in the center of a 3-$m/z$ unit wide mass selection window and applying low (5 V) collisional activation. The dotted lines show positions of the three precursor ions, and the stars represent the FT harmonics. The insets show isotopic distributions of the surviving precursor ions (right) and the “terminal” fragment ion, $\text{V}_{10}\text{O}_{25}^{-}$ (left). The precursor ion isolation window highlighted in orange and the black traces represent isotopic distributions of these ions in MS1.
In addition to the partial reduction of the molecular ions, another unexpected feature of the V10 mass spectra acquired in the negative ion mode is the presence of prominent ions representing neutral H₂O (and, to a significantly lesser extent, OH) loss from the molecular ions (Figure 1.1). Intriguingly, while the V₁₀O₂₆²⁻ ion consistently appears nearly equi-abundant to the doubly charged molecular V₁₀ ion, its singly charged counterpart (HV₁₀O₂₆⁻) is only a minor feature in the mass spectrum of V₁₀. Furthermore, it is the loss of two water molecules that gives rise to these oxygen-deficient V₁₀ anions. Oxygen loss from smaller oxovanadates (such as V₆) has been reported in the past, although the proposed scenario invoked a removal of a single oxygen atom from the metal oxide cluster upon addition of V(Mes)₃THF in dichloromethane, leaving behind a reactive V(III) within the molecule (inconsistent with the results of this work, where the most abundant signal in the isotopic clusters of V₁₀O₂₆²⁻ and HV₁₀O₂₆⁻ represents the fully oxidized species).

Earlier molecular modeling studies carried out with large oxovanadate clusters (V₂₀) concluded that it was the protonation of the surface oxygen atoms that weakened their interaction with the neighboring metal atoms and facilitated their removal from the cluster.

Although it might be tempting to conclude that the H₂O/OH loss from V₁₀ clusters observed in our work takes place in solution, one must remember that the extent of protonation of V₁₀ molecular species generated by ESI is significantly higher compared to that in solution (vide supra). Therefore, it is possible that the water abstraction process occurs in the gas phase, rather than in solution. In order to investigate the origins of oxygen-deficient V₁₀ species, tandem MS measurements were carried out on several molecular species of V₁₀ using low-energy collisional excitation (Figure 1.5). All parent ions displayed a remarkable degree of instability, with fragmentation ensuing immediately upon the precursor ion isolation even before any additional collisional activation is applied. Low-energy collisional activation results in robust dissociation of all precursor
ions, giving rise to the oxygen-deficient species, all of which had been observed in the mass spectrum of V10 prior to fragmentation. Interestingly, the isotopic distributions of the surviving precursor ions no longer show the presence of the partially reduced V10 species carrying “extra” hydrogen atoms (e.g., see the right-hand-side inset in Figure 1.5), which appear to be particularly vulnerable to collisional activation. In fact, it is the gas-phase dissociation of such partially reduced species that give rise to the “terminal” fragmentation products in Figure 1.5, e.g. V_{10}O_{25}^{2−}, which appears to be generated as follows:

\[
\left[H_{5}V^{(IV)}V_{9}^{(V)}O_{28}\right]^{2−} \rightarrow 2H_{2}O \rightarrow \left[HV^{(IV)}V_{9}^{(V)}O_{26}\right]^{2−} \rightarrow OH^{-} \rightarrow \left[V_{2}^{(IV)}V_{8}^{(V)}O_{25}\right]^{2−}
\]

The ease of generating the oxygen-deficient V10 ions (V_{10}O_{26}^{2−} in particular) provides strong evidence that they are MS artifacts. Further increase of collisional activation energy results in a dramatic expansion of the fragment ion repertoire by breaking the metal framework of the 

Figure 1.6. Zoomed views of mass spectra of V10 (sodium salt) acquired in H_{2}O and D_{2}O (black and blue traces, respectively) showing isotopic clusters corresponding to V_{10}O_{26}^{2−} (left) and V_{4}O_{16}^{2−} (right). The inset on the left panel shows the results of high-resolution measurements acquired in the narrow-band mode.

V10 cluster and giving rise to abundant V4 and V5 and less abundant V3 and V6 species (Figure 1.7), only one of which is observed in the original mass spectrum of V10 (vide infra).
Although the mass spectrum of V10 is dominated by decavanadate anions, two smaller (and lower-abundance) oxovanadate species, V$_6$O$_{16}^-$ and (H/Na)V$_4$O$_{11}^-$ are detected as well (Figure 1.1). While one of them (HV$_4$O$_{11}^-$) can be produced upon collisional activation of H$_4$V$_{10}$O$_{28}^{2-}$, its generation requires considerable collisional energy (Figure 1.7). V$_6$O$_{16}^{2-}$ was never observed in our tandem MS experiments (although its singly charged counterpart, HV$_6$O$_{16}^-$ has been detected at elevated collisional energy - see Figure 1.7). The absence of these ions among the fragments produced upon low-energy collisional activation of V10 anions suggests that they represent solution-phase oxovanadates. Furthermore, should they arise as a result of dissociation of metastable V10 species, one would expect a similar share of partially reduced species among them (e.g., V$_6$O$_{16}^{2-}$ should have an anomalous satellite peak M+1 corresponding to HV$^{(V)/V^V}$O$_{16}^{2-}$). However, the recorded isotopic distribution appears consistent with that calculated using the natural isotopic abundance, and acquiring the mass spectrum in $^2$H$_2$O does not result in a shift of the M+1.
peak to the $M+3$ position, as is the case for V10-derived fragments, such as $V_{10}O_{26}^{2-}$ (see Figure 1.6). Therefore, we conclude that $V_{6}O_{16}^{2-}$ and $(H/Na)V_{4}O_{11}^{-}$ represent smaller oxovanadate species that are present in solution alongside V10 (rather than being the products of its dissociation in the gas phase), and are not as susceptible to the partial reduction in solution under ambient conditions as V10 is.

A careful examination of V10 behavior using an array of MS-based methods presented in this work indicates that interpretation of straightforward MS measurements, particularly those carried out at lower resolution offered by conventional instruments, should be approached with extreme caution. Indeed, the additional protonation of V10 polyanions in the course of ESI process gave rise to metastable molecular ions. Water is readily abstracted from these metastable ions in the gas phase generating oxygen deficient V10 species. Furthermore, the apparent susceptibility of V10 to partial reduction in solution generates anions whose extent of protonation is even higher than that observed for fully oxidized V10 species, facilitating more extensive oxygen abstraction (via $H_2O/OH$ loss) in the gas phase. Although V10 dissociation gives rise to oxygen-deficient anions in the gas phase, rather than in solution, the underlying processes and the critical role of the surface-bound hydrogen atoms bear remarkable similarity to generation of “oxygen vacancies” within large oxovanadate clusters and indeed the bulk material surface that had been predicted in earlier computational studies. Oxygen vacancies are believed to be key to a range of catalytic properties of oxidovanadates, but their experimental investigation has been notoriously difficult, particularly on a larger scale. In this respect, high-resolution MS and MS/MS may lend themselves as potent tools capable of unveiling the most intimate details of oxygen vacancy formation within large vanadate clusters unaffected by solvent.
Decavanadate is a versatile member of the POM family displaying a variety of unique properties that can be exploited for a range of diverse applications. Our own interest in this molecule stems from its polyanionic nature that lends V10 as a viable candidate for applications in medicine as a disruptor of electrostatically-driven biopolymer interactions such as the platelet factor 4/heparin interactions underlying the etiology of heparin-induced thrombocytopenia. The unique chemical properties of V10 are also enjoying considerable attention in areas ranging from chemical catalysis to electronics. Last, but not the least, POMs in general and V10 in particular offer unique opportunities for fundamental studies in material sciences by serving as an atomically defined bridge between micro- and macro-scales.

Mass spectrometry, particularly ESI MS, has had long and illustrious history in inorganic structural analysis and related fields, although complex physical processes occurring within the ESI interface frequently require that the conclusions derived from such measurements be taken cum grano salis. For example, high-resolution measurements of ionic isotopic distributions allow the oxidation states to be determined at both single and multi-metal level but the measurement outcomes can be affected by the redox processes in the ESI interface. Likewise, mass measurements afforded by high-resolution MS provide a unique opportunity to unequivocally identify a wide range of metallo-compounds but can be complicated by the metastable nature of molecular ions giving rise to their dissociation in the gas-phase.

In this work we demonstrate that the origin of the redox processes affecting the oxidation state of POMs can be reliably traced to the bulk solution when incorporating ion exclusion LC as an on-line front-end separation tool in ESI MS measurements. We also use two orthogonal MS-based methods, tandem MS and hydrogen/deuterium exchange, to determine the origin of V10
species whose structures deviate from that of the “canonical” species. While this work is focused entirely on V10, the experimental strategies reported in this manuscript will undoubtedly advance the on-going efforts to elucidate structure and reactivity of other members of the POM family.
E: Bibliography


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CHAPTER II

DECAVANADATE INTERACTIONS WITH THE ELEMENTS OF THE SARS-COV-2 SPIKE PROTEIN HIGHLIGHT THE POTENTIAL ROLE OF ELECTROSTATICS IN DISRUPTING THE INFECTIVITY CYCLE


A. Introduction

Two years after being declared a pandemic by the World Health Organization, COVID-19\(^1\) shows no signs of abating, with the disease continuing to overwhelm healthcare systems worldwide, and the total number of fatalities already exceeding six million. The initial enthusiasm associated with the fast development and effective roll-out of the novel coronavirus vaccines\(^2\)\(^-\)\(^5\) has tapered off following the realization that their protection is not absolute,\(^6\) and durability is limited,\(^7\) mirroring the rapid decay of immune protection against recurring SARS-CoV-2 infections in former COVID-19 patients.\(^8\) The immune evasion problem has been exacerbated by the emergence and rapid proliferation of the novel variants of the SARS-CoV-2 in the second half of 2021, viz. Delta and Omicron.\(^9\) The multiple mutations giving rise to each novel variant also reduce the effectiveness of monoclonal antibody (mAb) therapies,\(^10\) diminishing available treatment options. At the same time, certain trends that are beginning to emerge from the analyses of SARS-CoV-2 mutations suggest that there may be alternative ways to design effective antiviral therapies. For example, multiple studies highlighted the anomalously high incidence of mutations that convert acidic or neutral residues of the SARS-CoV-2 spike glycoprotein (S) to basic ones both within the receptor-binding domain (RBD)\(^11\)\(^-\)\(^13\) and the furin cleavage site.\(^14\) The latter (particularly P681R - incorporation of an additional arginine residue within the PRRAR segment) is
advantageous from the point of view of the increased susceptibility of S to the proteolytic processing by the host enzymes critical for the viral particle’s ability to fuse with the host cell membrane. At the same time, the increased density of basic residues within RBD enhances docking of the viral particle to its host cell surface receptor angiotensin-converting enzyme 2 (ACE2) via electrostatic interactions with the low-pI ectodomain of this protein. This facilitates the initial anchoring of the virus in the extra-cellular matrix (ECM) by promoting stronger interactions with the polyanionic heparan sulfate chains of the proteoglycans, the major component of the ECM.13 In fact, several studies have pointed out at the electrostatic potential changes within S as the major factor responsible for the higher transmission rates of both Delta and Omicron.11,12

While the S mutations promoting a higher net positive charge within the key structural segments are advantageous vis-à-vis the ability of the virus to infect host cells, they also increase the pathogen’s vulnerability to therapeutic agents that act by disrupting the electrostatic interactions. Indeed, several reports pointed out that a highly anionic polysaccharide heparin, as well as some of its derivatives, may be effective at inhibiting the host cell entry by the SARS-CoV-2,15-17 and this polyanion has been already used in both treatment and prophylaxis of COVID-19.18-21 Although some of the therapeutic benefits of heparin in COVID-19 patients are due to its anticoagulant and anti-inflammatory properties, it is clear that its ability to interact with the key structural elements of SARS-CoV-2 S and block their association with the cell-surface receptors (such as the RBD/ACE2 binding22), also play an important role in mitigating the pathogenesis. Importantly, the long-range nature of the electrostatic interactions between polyanions (such as heparin) and S make them much less susceptible to the mutations within the latter. In fact, a range of mutations that gave rise to the Delta and Omicron (vide supra) would enhance the polyanion/S interactions, an effect that would be opposite to the antibody escape phenomenon that currently limits both the utility of mAb-based therapies and the extent of the acquired immune
Unfortunately, broader utilization of heparin-like polyanions in the COVID-19 therapeutic practice raises serious concerns, as this century-old anti-coagulant may induce internal bleeding in some patients, while triggering potentially deadly heparin-induced thrombocytopenia in others. These considerations motivate the search for alternatives that will be able to interact effectively with the positively-charged segments of S via long-range electrostatic forces that are less sensitive to the viral mutations compared to the short-range lock-and-key type of interactions, while at the same time lacking the properties of heparin that reduce the attractiveness of this polysaccharide in the COVID-19 setting.

Polyoxometalates (POMs) are a class of polynuclear inorganic compounds, which are mostly comprised of transition metal and oxygen atoms, although other atoms (such as phosphorus and silicon) may also be involved. Many POMs are polyanions, a property that endows them with a range of diverse biological activities (many of which can be potentially exploited for therapeutic purposes) that are mostly mediated by electrostatic interactions. In this work we explore the ability of decavanadate (V10, V_{10}O_{28}^{6-}), a paradigmatic member of the POM family, to interact with two positively charged structural segments of the SARS-CoV-2 S that play critical roles in the host cell infection (RBD and the furin cleavage site). We demonstrate that V10 not only interacts with both of these segments at physiological ionic strength, but in fact inhibits the RBD/ACE2 association, a key step in the cell infection process. Although V10 cannot be viewed as a viable antiviral agent due to its cytotoxic properties (as revealed by the in vitro studies with the SARS-CoV-2 infected cells presented in this work), its ability to interfere with the key processes underlying the infectivity of the novel coronavirus certainly warrants a more extensive search for novel and effective antivirals among other members of the POM family.
B. Methods

Materials. The recombinant forms of the receptor binding domains of RASR-CoV-2 S-protein (RBD) expressed in the baculovirus system and the ectodomain of the human angiotensin-converting enzyme 2 (ACE2) were purchased from Sino Biologics (Wayne, PA). The purity of both proteins was examined by intact-mass MS measurements as described earlier.22 The synthetic FCS model peptide (YQTQTNSPRRARSVAS, S residues 674-689, UniProt P0DTC2) was purchased from Biomatic (Cambridge, ON, Canada). The identity and purity of the synthetic peptide were verified by LC/MS/MS. All protein and peptide solutions were prepared in 150 mM ammonium acetate, pH adjusted to 7. Throughout all measurements, protein solutions were maintained at physiological ionic strength (150 mM ammonium acetate) to eliminate potential artifacts due to electrostatic interactions. V10 was synthesized as ammonium decavanadate salt by dissolving 2.997 g of ammonium metavanadate (Sigma-Aldrich, St. Louis, MO) in 100 mL of deionized water and titrating the resulting solution to pH 4 with formic acid and filtering the resulting solution. (NH₄)₆V₁₀O₂₈ was then precipitated by the addition of 150 mL of 95% ethanol and subsequently collected and washed via vacuum filtration. The product’s identity and purity were verified using mass spectrometry as described earlier.29 ESI MS was also used to verify the kinetic stability of V10 in aqueous solutions at pH 7 and relevant concentration (1-10 μM) for the time periods required for measuring V10/protein and V10/peptide interaction.

Mass Spectrometry. MS characterization of RBD and RBD/V10 interactions was carried out using a Synapt G2S (Waters, Milford, MA) hybrid quadrupole/time-of-flight mass spectrometer equipped with a nanospray source. Typical instrument parameters for native MS analysis were as follows: capillary, 1.3 kV; source temperature, 20°C; sampling cone, 120.00 V; extraction cone, 5.00 V; nanoflow gas pressure, 0.30 Bar. The mass calibration was carried out externally using perfluoroheptanoic. Processing of the raw MS data was carried out using the
UniDEC deconvolution algorithm, with the charge state assignment assisted by the limited charge reduction. Isolation of ionic populations in the trap cell for limited charge reduction measurements was performed by setting the quadrupole LM resolution values in the range of 4.3 - 4.7; and the gas phase polycation/anion reaction were triggered by introducing 1,3-dicyanobenzene anions after setting the trap wave height to 0.4-1.5 V. The anions were produced in the API source by setting the discharge current at 20 µA. Data fitting was performed with R-Studio using nonlinear least-squares fit with a convolution of Gaussian peaks ($dnorm$).

MS characterization of the FCS peptide and its interactions with V10 was carried out with a Solarix 7 (Bruker Daltonics, Billerica, MA) Fourier-transform ion cyclotron resonance (FT ICR) mass spectrometer equipped with a conventional ESI source and a 7 T superconducting magnet. All measurements were carried out in the positive ion mode using the following instrument parameters: ESI capillary, 4000 V; end plate offset, -500 V; nebulizer, 1.6 bar; dry gas, 4.0 L/min; dry temperature, 150 °C; capillary exit, 190 V; deflector plate, 190 V; funnel 1, 150 V, skimmer 1, 15 V; funnel RF amplitude, 200 V$_{p-p}$. The mass calibration was carried out externally using sodium formate as a calibrant.

**In vitro studies of V10 cytotoxicity and anti-viral properties.** In vitro studies were carried out by Miller laboratory at McMaster University. Vero E6 (ATCC CRL-1586) or U87mg-hACE2 cells were seeded in opaque 96 well flat-bottom plates (Costar) in complete DMEM (supplemented with 10% FBS, 1% L-glutamine, 100 U/ml penicillin-streptomycin, and 50mg/ml Geneticin for hACE2 cells). the media was replaced 24 hours post-seeding with complete DMEM containing indicated concentrations of V10 or solvent. 24 hours after pre-treatment, the media was replaced with SARS-CoV-2/SB3-TYAGNC$^{32}$ at a multiplicity of infection of 0.01, equalling to 300PFU/well with indicated concentrations of V10 or solvent diluted in low serum DMEM (supplemented with 2% FBS, 1% L-glutamine, 100 U/ml penicillin-streptomycin). The cells were then incubated at 37°C for
3 days (Vero E6 and U87mg-hACE2) before cell viability was determined. The plates were read by removing 50ul of culture supernatant and adding 50ul of CellTiter-Glo 2.0 Reagent (Promega) to each well. The plates were then shaken for 2 minutes, followed by reading the luminescence using a BioTek Synergy H1 or SpectraMax i3 microplate reader with gain of 135 and integration time of 1 second.

C. Results and Discussion

Consistent with the previous reports, C. native MS analysis of RBD exhibits a broad distribution of ionic signal featuring a significant overlap of ions corresponding to different charge states (Figure 2.1). Straightforward application of deconvolution algorithms to process MS data for such heterogeneous systems usually fails, and the charge state assignment was carried out by selecting ionic populations within narrow m/z windows followed by their brief exposure to radical anions. This process (known as limited charge reduction) allows well-defined charge ladders to be obtained, from which both ionic charges and masses can be readily calculated. Application of this technique to the mass spectrum of RBD reveals the presence of both the monomeric form of RBD (ionic signal within the m/z range 2,500-3,500; the average mass of 32.3 kDa) and a less abundant dimeric form of the protein in the m/z region above 3,500. The latter most likely arises due to formation of an external disulfide bond that involves a single unpaired cysteine residue within the RBD construct, as has been reported earlier. Addition of a nearly stoichiometric amount of V10 to the protein solution results in a noticeable change of the appearance of the mass spectrum (compare the blue and red traces in the Figure 2.1 inset). The
The convoluted appearance of this mass spectrum prevented application of common deconvolution procedures as a means of extracting mass distributions from the raw MS data (as was the case with free RBD – *vide supra*). However, the presence of inflection points within spectral features representing unique charge states indicates that at least two components contribute to the overall ionic signal (e.g., the ligand-free and ligand-bound forms of the protein). In order to verify that the latter is indeed an RBD·V10 complex, the ionic signal of RBD was modeled as a sum of normally distributed signals for all detected charge states:

\[
S_{\text{total}}^{\text{RBD}}(\mu) = \sum A_z^{\text{RBD}} \cdot e^{-\frac{(z+\mu z-M_0)^2}{2\sigma^2}},
\]  

(1)
where $A_z$ is the signal amplitude for the charge state $z$; $M_0$ and $\sigma$ are the average and the standard deviation of the protein mass distribution; and $\mu$ is the numeric $m/z$ value. The best fit was obtained by carrying out the minimization routine to optimize the values of $\sigma$ and $A_z$ (for $z = 10 - 13$). The lower charge states were excluded, since the corresponding ionic signals overlap with that of the dimeric form of the protein (vide supra). The results are presented in Figure 2.1 (bottom), with all charge states shown individually. Processing the mass spectrum of RBD acquired in the presence of V10 was carried out by assuming that the ionic signal representing the RBD-V10 complex is also distributed normally, and the standard deviation of this distribution is the same as for RBD alone:

$$S_{total}(\mu) = \sum \left( A_z^{RBD} \cdot e^{-\frac{(z+\mu_z-M_0)^2}{2\sigma^2}} + A_z^{RBD-V10} \cdot e^{-\frac{(z+\mu_z-(M_0+M_{V10}))^2}{2\sigma^2}} \right),$$

(2)

where $M_{V10}$ is the average mass of the electrically neutral form of V10, $H_6V_{10}O_{28}$ (963.4 Da). The data fitting results for the MS data acquired for RBD in the presence of V10 are presented in Figure 2.1 (top), with the simulated signals representing RBD alone and in complex with V10 shown in blue and red, respectively. The quality of the data fits can be further improved by modifying the intensity distributions for each charge state to account for the asymmetry (peak tailing towards higher $m/z$ values due to the incomplete desolvation of protein ions in the ESI interface). However, the extent of residual solvation depends on the charge state $z$, and the mathematical expressions for the ionic signal will include a significantly larger number of variable parameters compared to (1) and (2). As a result, nearly-perfect fits can be readily obtained using different sets of optimized parameters (i.e., in this case there is no unique solution to the optimization problem). Therefore, we restricted our calculations to the limited set of variable parameters, and the results are consistent with the notion of RBD interacting with V10 in solution giving rise to complexes of 1:1 protein:POM stoichiometry. Furthermore, the absence of the ionic signal
corresponding to the complexes of higher stoichiometry indicates that the RBD/V10 interaction is specific, as opposed to being a result of the non-specific adduct formation process occurring in the ESI interface.

The influence of V10 binding to RBD on the ability of the latter to associate with the SARS-CoV-2 host cell-surface receptor, ACE2, was studied by acquiring native mass spectra of an aqueous solution of a mixture of RBD (2.9 μM) and the recombinant form of the ectodomain of human ACE2 (1.9 μM) at physiological pH/ionic strength both in the presence and in the absence of V10. The RBD/ACE2 mass spectrum features an abundant signal within the m/z range 6,500-8,000 with partially resolved charge states (Figure 2.2A). Fitting this distribution using an approach similar to that outlined above (i.e., using equation (1), mutatis mutandis) without fixing the $M_0$ value gives rise to a range of charge states (see the purple color-filled curves in Figure 2.2A) and the average mass value of 248.3 kDa, consistent with the previously reported mass of the RBD2ACE2 complex²² (since the ectodomain of ACE2 forms a stable dimer under near-native conditions, which can partially dissociate under certain conditions, we will use the ACE2₂ notation to designate the canonical dimeric form of this protein in order to avoid confusion). Addition of V10 to the RBD/ACE2 mixture (to a final concentration of 5 μM) results in a noticeable change of the appearance of the mass spectrum in the high m/z region (Figure 2.2B). The ionic signal shifts
to a slightly lower m/z region (6,000-7,000) and processing this signal using the data fitting procedure outlined above yields notably lower values of the charge states and the average mass (184.0 kDa), consistent with the mass of ACE2 free of RBD. Importantly, no signal corresponding to a putative partially saturated RBD-ACE2 complex could be detected, consistent with the notion of a complete dissociation of RBD from ACE2.

Evaluation of V10 interaction with the furin cleavage site of the SARS-CoV-2 SGP was carried out using a peptide YQTQTNSPRRARSVAS (SGP residues 674-689, UniProt P0DTC2), which incorporates the arginine-rich segment recognized by serine proteases. The high-resolution mass spectrum of this peptide (labeled FCS in Figure 2.3) undergoes a notable change upon addition of a small molar excess of V10 to the peptide solution. In addition to peaks representing the unbound
forms of the peptide (charge states +2 and +3) and V10 (H₂V₁₀O₂₈⁺ and NH₄H₂V₁₀O₂₈⁺, the positive charge states of which are expected and consistent with the previous work²⁹), a prominent signal is observed at m/z 929. The experimentally measured monoisotopic m/z value for this ion is 929.0908, a number that is within 7 ppm of the calculated monoisotopic m/z value for the +3 charge state of the FCS·V₁₀ complex ion (929.0967). These measurements were carried out at physiological ionic strength to eliminate non-specific electrostatic interaction in solution. In addition to the +3 charge state of the FCS·V₁₀ complex, a weaker ion signal was detected for the +2 charge state (the measured monoisotopic m/z value 1393.1311 vs. the calculated one of 1393.1414). Minor signals were also detected for the FCS₂·V₁₀ (charge state +4, monoisotopic m/z 1152.3001) and FCS·V₁₀₂ complexes (charge state +3, monoisotopic m/z 1255.8000).

Increasing the concentration of V₁₀ in solution (up to a 5-fold molar excess over the model peptide) resulted in an increase of the relative abundance of ions representing the FCS·V₁₀ complex, but ions corresponding to the free peptide were always present in the mass spectra (data not shown).

Evaluation of the anti-viral activity of V₁₀ was carried out in Miller laboratory at McMaster University using both human U87mg-hACE2 and Vero E6 cells. The cells were challenged with SARS-CoV-2 (300 PFU/well), resulting in a significant decrease of their viability (Figure 2.4). Addition of V₁₀ to the infected cell cultures did not result in any noticeable increase of the cell viability at levels as high as 1 μM. In human cells, a transient viability increase was observed above 1 μM, but was immediately followed by a precipitous decline (Figure 2.4A). This dramatic decrease in the cell viability at 10 μM and above mirrored behavior of the uninfected cells, which clearly manifested V₁₀ cytotoxicity in that concentration range. The cytotoxicity could be clearly ascribed to V₁₀, rather than other components of the mildly acidic V₁₀ solution used in these
measurements, as no change in the cell viability was detected when both virus-challenged and healthy (uninfected) cells were treated with the equivalent amounts of the “blank” (a solution having identical composition to the V10 stock solution, but lacking the inorganic polyanion), as shown in Figure 2.4B. The Vero cells also exhibited remarkable susceptibility to V10 at doses exceeding 10 μM (Figure 2.4C), while remaining insensitive to other components of the V10 solution (Figure 2.4D).
The major route of the SARS-CoV-2 entry into the host cell (Figure 2.5) exploits the ability of S to associate with ACE2, which is ubiquitously expressed on the surface of many cell types. The electrostatic forces not only play an important role in the interaction between the RBD of the SARS-CoV-2 S with ACE2 (which has a theoretical pl of 5.36), but also in the initial encounter of the virus with the host cell, which is mediated by the heparan sulfate (HS) proteoglycans. The critical dependence of the viral docking on the electrostatic interactions suggests that it can be potentially exploited for therapeutic purposes. In fact, the success of polyanionic biopolymer heparin and heparin-derived medicines in mitigating the pathological consequences of COVID-19 infection is attributable, at least to some extent, to their ability to associate with RBD and prevent its interaction with both HS proteoglycans and ACE2. Native MS (Figure 2.1) provides convincing evidence that V10 has the ability to associate with RBD in solution under physiologically relevant conditions (neutral pH and physiological ionic strength), which is not surprising given its polyanionic nature. However, the ability of V10 to associate with RBD does not necessarily mean that it would diminish the affinity of the latter for the cell-surface receptor. Indeed, there are two well-defined and extended positive-charge patches on the surface of RBD (Figure 2.6), where V10 can occur due to significant electrostatic attraction, but only one of them has a significant overlap with the receptor-binding motif. Nevertheless, the native MS
characterization of the RBD/ACE2 interaction clearly indicates that the presence of V10 in solution results in a facile disassembly of the RBD/ACE2 complex (Figure 2.2). This behavior mirrors the effect of short heparinoids (such as the highly anionic pentasaccharide fondaparinux) on RBD, which form only 1:1 complexes with the protein but nonetheless effectively disrupt its interaction with the receptor.22,40 Interestingly, the results of the earlier molecular modeling work in the case of RBD/short heparinoid association suggest that the polyanion binding to the protein occurs outside of its receptor-binding motif; nonetheless, the ensuing conformational changes rearrange the latter allosterically to an extent that is sufficient to disrupt the RBD/ACE2 interaction.22 It is possible that the V10 interference in the RBD/ACE2 interaction follows a similar scenario, although native MS alone obviously cannot provide atomistic details of this process. What is clear is the fact that this interference is sufficiently effective, highlighting the potential of V10 to act as a disruptor of the virus docking to the cell surface, although it is important to remember that the avidity of the S/ACE2 interactions (i.e., binding of a single virion particle to multiple receptors on the cell surface) could influence the efficacy of V10 differentially in an infection model relative and an in vitro binding assay.
Another critical step in the viral cell entry that can be potentially inhibited by electrostatic interactions is the proteolytic processing of the SARS-CoV-2 S by the cell-surface proteases. Proteolytic processing of the SARS-CoV-2 S makes its fusion peptide (localized in the S2 domain of the protein close to the segment connecting the S1 and S2 domains) available for anchoring into the cell membrane, which is followed by fusion of the virus with the cell, a mechanism common to all coronaviruses (Figure 2.5). The two main actors (furin and the transmembrane serine protease 2, or TMPRSS2) recognize two distinct sites within the segment connecting its S1 and S2 domains. While the activation of S fusogenic activity is performed by TMPRSS2 (which cleaves the SGP polypeptide chain at the so-called S2’ cleavage site on the N-terminal side of the fusion peptide), this step cannot be completed without the S priming by furin. The latter processes the S polypeptide chain at the so-called S1/S2 cleavage site, which incorporates the polybasic RRAR furin recognition element. Although furin is a part of the host cell secretory machinery, and is believed to process the nascent S chains inside the cell upon the virus’ hijacking the cellular protein expression system, it can also be present on the cell surface and even released into circulation. In fact, the presence of furin on the cell surface is required for toxin activation and cell entry by a range of bacterial pathogens. Therefore, it is reasonable to assume that at least a fraction of the SARS-CoV-2 S are processed by furin either on the cell surface or while in circulation. Furthermore, several other blood-borne serine proteases, such as factor Xa and thrombin, can also process S, enhancing the viral cell entry and exacerbating infectivity.

The polybasic furin cleavage site is located within S has anomalous degree of conformational motility as predicted by multiple algorithms comprising the PONDR (Predictor of Natural Disordered Regions) engine (nor is this segment visible in the crystal structures of this protein). Therefore, a polypeptide with an amino acid sequence corresponding to this unstructured region can be used as a realistic model of this S segment (residues 674-689,
YQTQTNSPRRARSVAS, which we refer to as FCS). The arginine-rich part of this sequence endows FCS with a significant positive charge, which had been shown to have affinity to anionic biopolymers, such as heparin. The polyanionic nature of V10 suggests that it may also interact with the arginine-rich segment of the FCS, and the mass spectrum of this peptide acquired in the presence of V10 (Figure 2.3) indicates that the two oppositely charged molecules do form a complex in solution despite the charge-screening effect of the salt. It remains to be seen to what extent the proteolytic processing of FCS is inhibited by this interaction; nevertheless, it seems plausible that binding of a bulky inorganic molecule, such as V10, to the protease substrate right at the cleavage site would interfere with the proteolysis. At the same time, we note that the presence of the abundant signals of the free peptide and V10 alongside the FCS-V10 complexes in the mass spectrum shown in Figure 2.3 indicates that the interaction is not very strong. Although no affinity measurements were performed in this work, an estimate based on the total FCS and V10 concentrations, as well as the relative intensities of the signal suggest the $K_D$ value for this system exceeds the $10^{-5}$ M level. Although this represents transient interaction within the peptide/POM system, it may interfere with the enzymatic processing of the furin cleavage site given sufficient concentrations of the polyanionic ligand.

The results of the experimental work discussed so far provide a clear indication that V10 has a capability to interfere with the key steps of the SARS-CoV-2 infectivity cycle. While the conclusions of the studies carried out with the model proteins and peptides are encouraging, they do not provide evidence that SARS-CoV-2 entry can be inhibited in living cells using the physiologically reasonable (safe) concentrations of the inhibitor. The latter can be verified only using in vitro models, and unfortunately the cell culture work indicates that V10 is cytotoxic at concentration levels above 1 μM (Figure 2.4). Interestingly, these studies also indicate that V10 may begin to manifest its antiviral properties right before the onset of the cytotoxicity. Although
one might see the results of the in vitro work presented in Figure 2.4 as disappointing, as V10 fails to effectively inhibit viral infectivity at safe (sub-cytotoxic) levels, further work targeting regions of SARS-CoV-2 S protein vulnerability with inorganic polyanions is certainly warranted. Our work demonstrates that electrostatic forces can be exploited to interfere with the interactions between the virus and its physiological targets, and the enormous chemical and structural diversity exhibited by POMs remains an untapped source of potential antivirals. Indeed, the therapeutic potential of this class of inorganic molecules is actively explored in areas ranging from oncology to antibiotics, while their anti-viral activity received relatively little attention. While our work focused on the ability of one specific vanadium-based POM (V10) to target the key steps of the SARS-CoV-2 entry into the host cell (Figure 2.5), vanadium exhibits a range of other biological activities, such as anti-inflammatory and anti-hyperglycemic effects, that are also beneficial vis-à-vis mitigating clinical manifestations of severe COVID-19.

D. Conclusions

The relentless search for the effective and safe therapeutic treatments of COVID-19 continues to suffer setbacks due to the rapid evolution SARS-CoV-2. While the antiviral agents designed using the traditional lock-and-key approach may be rendered ineffective by a single mutation, exploitation of the less specific/longer-range electrostatic interactions as an alternative therapeutic strategy may prove more robust. Heparin and related highly anionic polysaccharides had been actively investigated in this regard since the beginning of the pandemic, and have already entered clinical practice despite some documented shortcomings. At the same time, several other classes of electron-rich compounds remain largely overlooked, including inorganic polyanions such as POMs. Investigation of V10, a paradigmatic member of this family, vis-à-vis its ability to interfere with the key steps of SARS-CoV-2 entry into the host cell highlights the potential of POMs as effective disruptors of both the viral particle docking to the cell surface receptor
(ACE2) and the following proteolytic processing required for activation of the fusogenic activity of the virus. Although the in vitro studies identify the cytotoxicity of V10 as a major factor limiting its utility as an antiviral agent, the collected data provide compelling evidence that the search for safe, effective and robust COVID-19 therapeutics among the members of the POM family is warranted.
E. References


A: Introduction

Isotopic exchange has been widely utilized experimentally for a variety of analytes, and Hydrogen Deuterium Exchange and its ever-increasing presence in the field of protein analysis is one of the most prevalent of its contemporaries. When combined with mass spectrometry, the ability of isotopic exchange to record a transient kinetic event as a physical property in the form of a mass shift is instrumental in our ability to study these otherwise elusive occurrences.\textsuperscript{1} HDX-MS has allowed unique events in protein dynamics to be studied down to a residue level.\textsuperscript{2-3} While this may be one of the largest fields of interest around isotopic exchange, the information that can be obtained from isotopic exchange analysis is useful to a much wider field of analytes. One such class of molecules is that of the Polyoxometalate (POM), clusters that exist in between highly order bulk oxometalate solids and smaller solution phase species, combining structural elements of the former with the solubility of the latter.\textsuperscript{4} Increasingly large and complex POMs are being generated with a wide array of catalytic, biomedical, and material use cases.\textsuperscript{5-7} Of note is the POM Decavanadate (V10), which is particularly interesting to the field for its varied biomedical and catalytic interactions.\textsuperscript{8-9} Recent analysis has revealed that some V10 exists in solution in a partially reduced state, rather than being entirely oxidized as previously believed (Chapter I).\textsuperscript{10} The complex speciation of oxovanadates indicate that the solution phase dynamics of V10 merit further investigation via isotopic exchange.

Isotopic exchange of V10 has been studied previously via mass spectrometry in a limited capacity.\textsuperscript{11} The incorporation of \textsuperscript{18}O into V10 was previously studied using mass spectrometry by incubating a solution of V10 prepared in \textsuperscript{18}O enriched water and precipitating out small samples
at specific times using CsCl. The oxygen in the resulting samples then evolved to CO₂ which was then studied via mass spectrometry. The exchange profile observed corresponds to the average rate of incorporation across all molecules and oxygen atoms. In many ways this experiment parallels Lindstrom-Lang’s method of cryosublimation analysis of protein deuterium content, and more closely resembles that than any modern HDX-MS experiment. These early isotopic exchange measurements, while revolutionary for the time, were naturally limited in that there was no structural or molecular specificity to the measurements. NMR measurements conducted on Decavanadate and Decaniobate (structurally similar to V₁₀ save for the substitution of Vanadium centers with Niobium) demonstrate how NMR can overcome the lack of structural specificity and obtain detailed information on the exchange rate of structurally unique oxygens, but lacks the context of how these relate to the level of exchange of any whole molecule. Other POMs have been analyzed using oxygen exchange coupled with mass spectrometry, but the complex isotopic distributions observed prevented in-depth analysis of the data.

HDX-MS measurements performed using high-resolution mass spectrometry demonstrate the capabilities of modern isotopic exchange analysis. In the case of proteins this can let us distinguish between conformers of a protein by is relative level of exchange. Furthermore, kinetic information on these states can be gleaned from the resulting data. Proteins and inorganic complexes are dramatically different molecules in terms of both composition and behavior, but they possess several shared qualities that make them well suited to this type of analysis including label atoms capable of exchanging with their counterparts in solution. In the case of global HDX and oxygen exchange this leads to the formation of distinct populations of the analyte that can be resolved in the m/z domain and can be interpreted as metastable states and global unfolding/dissociation events.
In the work presented in this chapter we utilize oxygen exchange analysis to study possible metastable configurations of V10. From that data we can confirm the existence of these metastable states which can be observed as distinct populations in the oxygen exchange profile. Fits of the populations observed can be used to determine the kinetics of the metastable state being sampled. This data combined with a comparison of different exchange conditions can be used to generate a model exchange pathway that could explain the complex dynamics of V10 in solution.

**B: Method**

V10 was synthesized by dissolving ~ 3 g of ammonium metavanadate in deionized water and adjusting its pH to 4.5 using formic acid. V10 was collected as a solid by adding 150 ml of cold ethanol and vacuum filtering the resulting precipitate. The oxygen exchange experiment was conducted by first preparing a solution of $^{18}O$ substituted V10 using $^{18}O$ enriched water (Cambridge Isotope Labs, 98.2% enrichment) acidified with 0.08% formic acid by volume. The sample was incubated until V10 was saturated with $^{18}O$ (as determined via mass spectrometry). The aliquots of the sample were subsequently diluted into the specified sample conditions with measurements taken at the described timepoints. All spectra produced in this experiment were acquired on a Bruker Solarix 7T FT-ICR-MS in narrowband mode. Typically instruments parameters were capillary voltage of 4000 V, spray shield: -500 V, drying gas temperature: 150°C, ion accumulation time: 0.245 seconds, and Q1 mass: 100 m/z. Subsequent processing of the data was performed using RStudio and related packages.19-26

**C: Results and Discussion**

As reported previously, V10 appears in mass spectra alongside loss-of-water fragments, adducts, and partially reduced species, complicating analysis of oxygen exchange MS data.
The mass spectra generated are further complicated by the now extended isotopic distributions of these populations, resulting in heavily convoluted spectra. Differences in mass between observed species on the order of 1-10 mDa are common, and if peaks are not fully resolved then extracting accurate abundances for any specific isotopic composition would be impossible. As such, the mass spectra utilized for these measurements were acquired in the Solarix’s narrowband mode to achieve a sufficient resolution ($R \approx 9.3 \times 10^5$, based on FWHM) to overcome this. Direct analysis of the spectra generated demonstrates that we can successfully resolve these nearby species (Figure 3.1). Peaks were grouped across species and each replicate at each timepoint was normalized to one such that the resulting plots show the fractional amount of V10 for any given level of $^{16}$O incorporation. Unlike HDX, back exchange is only a minor problem since samples are analyzed at the timepoint listed without an intervening gap or quench step. As such the only potential back exchange that can occur comes from the ~0.983% $^{18}$O present after

Figure 3.1. Narrowband mass spectrum oxygen exchange of 40 uM Decavanadate at 6 hours showing distribution of $\text{V}_{10}O_{26}^{2-}$ (red) and $\text{H}_4\text{V}_{10}O_{28}^{2-}$. Inset figure shows a zoomed view of region in which the extended isotopic distributions of V10 species overlap.
diluting the enriched V10 solution, which should be negligible given the overall slow rate of exchange. At ambient temperature the exchange goes to completion on the order of days, varying with respect to the solution phase conditions (notably the pH of the solution).

On inspection of the data, it was observed that multiple populations of V10 exist in solution, each interacting uniquely with the solvent yielding different levels of $^{16}$O incorporation (Figure 3.2). The population with the lowest level of exchange was thought of as intact V10, owing
its incorporation of $^{16}$O to the slow exchange of the solvent exposed oxygen atoms. This describes the baseline level exchange expected at any timepoint from which we can infer that any species showing a higher level of $^{16}$O incorporation has undergone some conformational or structural change. In HDX terminology this species conforms most closely to the uncorrelated exchange regime. In protein dynamics uncorrelated exchange describes either a scenario in which either the rate of a larger dynamic event (such as the sampling of a metastable folding conformation) is significantly higher than the rate of hydrogen exchange or a scenario in which the dynamic event only encompasses one surface exposed hydrogen.\textsuperscript{17} Whereas for V10 it is likely that this population does not undergo a dynamic event that impacts oxygen exchange. Instead, the apparent uncorrelated presentation is indicative of the slow exchange rate of surface oxygens.\textsuperscript{15} The most exchanged population matches the final isotopic ratio of the solvent ($\sim$99 % $^{16}$O), indicating that this population’s oxygen has been completely exchanged. This population appears to undergo correlated exchange in which the kinetics of the exchange event are significantly faster than the related dynamic event of the molecule.\textsuperscript{17} In POMs it is likely that this corresponds to some dissociation and subsequent reconstitution of the POM complex, which is in line with the previous studies showing that the rate of oxygen exchange for smaller oxovanadates such as VO$_4^{3-}$ is on the order of seconds.\textsuperscript{27}

The last groups observed exist in-between the intact and dissociated populations, exchanging somewhat like both the correlated and uncorrelated regimes as it slowly resolves itself from the intact population. This population behaves like a metastable state, akin to those observed in protein structure. Like in proteins these states can be transiently sampled with lifetimes too short for traditional analysis. When these states are accessed, they enable some additional level of exchange, accelerating the exchange rate of the intact conformation without reaching the terminal population. At times two distinct populations corresponding to metastable
states can be observed, although it is not known whether this corresponds to two distinct events or the same event occurring independently at two different regions in V10’s structure. The emergence of these metastable states in proteins is attributed to the numerous folding conformations that can be accessed by the protein and can be attributed to unfolding of specific regions of the protein or conversion to a metastable conformation. These conformational changes are now known to play an important role in a broad range of protein activity. POMs, as demonstrated by V10, lack both things being significantly smaller and less complex with their near-crystalline arrangement of atoms. Yet they display dynamics with a degree of complexity like that of biopolymers, and these unique conformations may have similar implications for POMs as the metastable protein configurations had for proteins. These observations hold when looking at

Figure 3.3. Gaussian peak fits (green, brown, and purple) and Binomial fit (pink) of V10 populations at 10 hrs (c). Models of intact V10 (a), and proposed metastable states (b,d).
partially reduced V10, with little to no difference between fully oxidized V10 and V\textsuperscript{V}\textsubscript{9}V\textsuperscript{IV} indicating that the rate of interconversion between oxidized and reduced V10 is fast enough relative to the rate of exchange that the resulting data is an average of the two states (Figure 3.4).

Prior NMR studies of oxygen exchange for V10 and related decametalates have likewise suggested the presence of these metastable states and led to theories of their identity. The proposed
metastable states revolve around two intermediate structures, a V6 cluster and a V4 ring that can emerge via either complete or partial dissociation of the V10 structure (figure 3.3b, 3.3d).\(^{14-15, 27}\) Sampling a less compact metastable state allows easier access to otherwise occluded oxygen atoms. Additionally, the less rigid V4 ring produced would likely have significantly more labile oxygens, speeding up the exchange rate. Furthermore, the sampling of these states is dependent on two solvent oxygens attacking vanadium centers which may be incorporated into the V10 structure once it closes. The second smaller metastable state that emerges at the 8-to-10-hour timepoint likely corresponds to V10 molecules that have sampled the same metastable state again on the opposite side, resulting in a similar additional shift. The V4 ring that emerges from the metastable state closely resembles a canonical structure of \(\text{V}_4\text{O}_{12}^{4-}\) that has been shown to exist natively in solution.\(^{29-30}\) The V6 remainder has no such analogue and is likely unstable. In the case where the V4 ring fully dissociates from the V6 cluster it is possible that the V6 undergoes further dissociation, into either V4 and V2 or two V3 molecules, all of which are canonical solution phase species.\(^{30}\) This is likely the complete dissociation that leads to the correlated exchange of the completely exchanged terminal population.

**Figure 3.5.** Average area of V10 populations over time for pH 4.0 and pH 4.5 at 20°C.
Subsequent experiments focused on determining the effect of both pH and temperature on the exchange process. The conditions were identical save for adjusting either the pH or temperature under which the exchange occurred. Prior computational research has indicated that the global exchange process is acid catalyzed.\textsuperscript{31} Hydrogen binding to exposed oxygen in the POM structure weakens the bond of the oxygen with its corresponding Vanadium atoms allowing for

Figure 3.6. Oxygen exchange profile of $\text{H}_2\text{V}_{10}\text{O}_{28}^3-$ at pH 4.5 and ambient temperature.
abstraction of the oxygen. The prediction trend can be seen in the experiment conducted at pH 4.5, which shows an overall lower level of incorporation for the non-fully exchanged species at any given timepoint (Figures 3.6, 3.7). To better observe the relative abundance of the previously observed populations, gaussian peaks and binomial distributions were fit to the data. Populations were fit using gaussian peaks except in the case of the fully exchanged population, which fits a
binomial distribution corresponding to the purity of $^{16}$O in the final sample (Figure 3.5c). From the area of the fits, we can find the relative abundances of all the populations at each timepoint. In cases in which the metastable state populations could not be fit, their area was calculated by finding the difference between the sum of the areas of the other states and the total area.

A comparison of all the experimental conditions used reveals that the exchange profiles of V10 are heavily dependent on the solution pH and temperature. Higher temperature accelerates the process by increasing the rate at which all populations incorporate oxygen, as well as by increasing the rate at which the metastable states and the dissociated state are sampled (Figure 3.8). Even under these conditions the rate at which the dissociated state is sampled outpaces the baseline rate of oxygen exchange (i.e. the majority if not all of the V10 in the fully exchanged population accesses it through dissociation). Generally, it appears that lower pH and

Figure 3.8. Average area of V10 populations over time for pH 4.5 at 30°C.
higher temperatures yield higher rates of sampling of both the metastable and dissociated states. In the case of temperature this trend is as expected. Speciation diagrams for oxovanadates in aqueous solution indicate that it should be more stable at pH 4 than 4.5, and as such if we attribute these transient states to complete and/or partial dissociation, we would naturally expect them to be sampled more frequently under conditions in which the complex is less stable. The experimental data collected suggests the opposite, that the sampling of these states is at its highest under conditions V10 is stable at. This raises questions about the nature of these dissociation processes, namely whether they are tied to stability, and if so, do they instead play a role in maintaining V10 stability rather than undermining it. If so, it could be that sampling these metastable states prevents dissociation by introducing flexibility to the V10 complex.

The relative proportion of each population present at each timepoint can also be used to obtain kinetic information about the sampling of the metastable and dissociated states. Accurate modeling of the entirety of the exchange process would require oxygen specific exchange rates for each structurally unique oxygen across all populations, so an abstracted approach is needed. As such we thought of the kinetics in terms of the stable population (S), the sum of the metastable state populations (M), and the dissociated population (D). From this we can broadly conceive of

![Figure 3.9. Comparison of kinetic models (dashed trace) for V10 oxygen exchange at pH 4. Models shown either treat the metastable state as an intermediate between the stable and dissociated states (a) or treat the metastable state and dissociated state as kinetically unrelated (b).]
two potential schemas for kinetics of the exchange process. The first is a scenario in which the
dissociated state is sampled independently from the metastable state, in which the relative
proportion of S and M would not affect the kinetics of D. The rates for this setup could be described
in simple terms by equations 1-3 in which k1 is the rate constant for the sampling of the metastable
state and k2 is the rate constant for the sampling of the dissociated state.

\[ S \rightarrow M \quad (k1) \]

\[ S/M \rightarrow D \quad (k2) \]

\[ S'[t] = -k1 * S[t] - k2 * S[t] \quad (1) \]

\[ M'[t] = k1 * S[t] - k2 * M[t] \quad (2) \]

\[ D'[t] = k2 * M[t] + k2 * S[t] \quad (3) \]

When solved for S[t], M[t], and D[t] (performed using DSolve in Mathematica) we can then
fit those functions to the areas of the previous fits and acquire k1 and k2 (for S[t] we can only fit
the sum of k1 and k2 and not each individually). From this we observe accurate fits for S[t] and
M[t], but the fit acquired for D[t] deviates significantly from the data acquired experimentally
(Figure 3.9.b.). In this schema we expect the observed rate of sampling of D to be at its maximum
at t = 0 because that is the point at which the total abundance of S and M are at their highest.
Whereas in the experimental data the rate is at its highest at approximately 6-8 hours. As such an
alternative schema may better fit our data. In the second scenario the S population can not directly
sample the D state, instead M serves as an intermediate between S and D. As such the rate of
formation of D is governed by the concentration of M exclusively as shown in equations 4-6. Fitting
the solved S[t], M[t], and D[t] functions results in a much more accurate fit for D[t], wherein the
maximal rate of formation of D coincides with the maximal relative amount of M (Figure 3.9.a.).

\[ S \rightarrow M \quad (k1) \]
\[ M \rightarrow D \quad (k2) \]

\[ S'[t] = -k1 \ast S[t] \quad (4) \]

\[ M'[t] = k1 \ast S[t] - k2 \ast M[t] \quad (5) \]

\[ D'[t] = k2 \ast M[t] \quad (6) \]

While it is unlikely that these rate equations capture all the factors contributing to the rate of sample of these populations, the difference in apparent accuracy of these fits indicates that the sampling of the metastable state in solution by V10 is a precursor to a larger dissociation event. Although the sampling of these states is transient, the lingering structural effects of this process either destabilize the complex or otherwise enable the dissociation of the V10 complex.

When studied in its totality the oxygen exchange data for V10 indicates two potential exchange pathways. The first is a scenario in which the V10 center exchanges through a combination of slow incorporation of solvent oxygen at the V10 surface and a sampling of metastable states (figure 3.10, red background pathway). This pathway requires sampling the proposed metastable state twice to reach near complete exchange. Notably this pathway also features scrambling of the oxygen in the V6 cluster due to the possibility of the V4 ring swapping to a different side of the complex than it first detached from. The resulting scrambling is also a way for otherwise inaccessible core oxygen to exchange. A surface-exposed oxygen at the V10 surface exchanges, and then during a subsequent metastable state moves to the interior position.

The second pathway is a large-scale dissociative event that most likely follows from the V4 ring fully detaching from the V10 complex (figure 3.10, blue background pathway). Given that this event results in complete exchange (the binomial fit yields a probability in line with the expected abundance of \(^{16}\)O in the final solution) the V6 complex either exchanges oxygens at a significantly
faster rate than V10 or deteriorates down to the metavanadate level wherein the exchange is completed in seconds.\textsuperscript{27}

\textbf{D: Conclusion}

Oxygen exchange analysis coupled with high-resolution mass spectrometry is a powerful tool for the analysis of the dynamics of POMs in solution. The results of the analysis of V10 using this technique allows for the observation of previously theorized metastable states. Furthermore, we can compare experiments across solution phase conditions to determine the effect of temperature and pH on the V10 complex, showing that lower pH and higher temperatures lead to faster sampling of the metastable and dissociated states. The areas of the populations observed can be used to gather kinetic data about the reaction. From this kinetic information we can show that the metastable state serves as an intermediate for a larger dissociation event. The ability to study otherwise difficult to observe metastable states and other transient events makes oxygen exchange analysis a useful tool in the study of POMs going forward.


CHAPTER IV

INVESTIGATION OF CAGE-LIKE POV SPECIES.

A. Introduction

In contrast to the condensed structure of V10, there also exist cage-like POVs which possess several distinct properties. While we demonstrated that V10 is capable of having reduced V centers, the cage-like species have significantly more heterogeneity in terms of the oxidation states of its constituent vanadium atoms containing a significantly higher proportion of V<sup>IV</sup> than previously observed in V10<sup>1-3</sup>. These structures typically surround a heteroatom that is either free floating (for halogens like Cl<sup>-</sup> and other non-oxide molecules like N<sub>3</sub>−) or bound to the POV structure (for oxides like VO<sub>4</sub><sup>3-</sup>)<sup>4-6</sup>. It has been shown that the nature of the payload determines the overall structure of the POV species with even small differences resulting in large changes to the overall POV structure<sup>7</sup>. There is also a significant structural difference in the geometry of the oxovanadate subunits, which typically have a square-pyramidal geometry rather than the octahedral geometry seen in V10<sup>5</sup>. This square pyramidal geometry results in the exposure of the vanadium center to the inside of the cage allowing for interaction between the sixth coordination site of the vanadate and the anionic payload<sup>8</sup>. Each vanadate subunit has a terminal outer facing oxygen, with the remaining oxygens in a μ<sub>3</sub> (and μ<sub>2</sub> occasionally) coordination bridging the vanadium atoms<sup>9</sup>.

A notable quality of cage-like POVs is the wide array of molecules that can serve as a payload. Common traits shared among POV payloads include an overall negative charge (some are also neutral), small size/mass relative to the overall POV structure, and a degree of symmetry around which the cage POV structure can be formed<sup>2, 7</sup>. The ability of POV structures to adapt themselves to their payload has made them a subject of investigation in a wide array of fields both for the unique structures that can be generated and for the specific payloads that can be packaged.
Notably they have been used for NO\textsubscript{x} sensing, anti-cancer, and activation of GPCR\textsuperscript{10-12}. The template like assembly allows for the generation of chiral nanocages, and anisotropic nanocages\textsuperscript{13-14}. They also can offer the ability to shield a redox sensitive payload by being reduced or oxidized in their place\textsuperscript{15}. The adaptability of these molecules is such that they can even conform to a new payload after the initial synthesis, with H\textsubscript{3}V\textsubscript{19}O\textsubscript{50}\textsuperscript{8-} (VO\textsubscript{4} centered) structures being capable of spontaneous encapsulation of N\textsubscript{3} on subsequent heating\textsuperscript{16}.

As demonstrated in the first chapter, POVs can display significant heterogeneity in observed oxidation states in solution with multiple species coexisting in solution. Cage-like POV structures have been shown to range from predominantly fully oxidized V\textsuperscript{V} centers to being entirely V\textsuperscript{IV} centers\textsuperscript{2}. Determining the relative proportion of these oxidation states is an important quality when identifying these species as the delocalized nature of the additional electrons from V\textsuperscript{IV} can influence the larger cage structure\textsuperscript{15}. Additionally, the delocalized electrons in reduced POV nanocages can yield unique magnetic properties\textsuperscript{17}. Mass spectrometry, as demonstrated previously, is able to differentiate POMs with distinct oxidation state profiles due to the corresponding change in charge or mass (due to the addition of charge-balancing hydrogen atoms)\textsuperscript{1}. This ability to characterize POV cages both by mass and charge makes MS analysis particularly well suited to the speciation of these complex inorganic molecules.

Previously reported synthetic pathways for POV cages mainly feature hydrothermal and related techniques wherein the precursor material is heated for a prolonged period, and potentially under elevated autogenous pressure\textsuperscript{18-19}. Such synthetic pathways typically take one or more days, and commonly require a sealed reaction vessel and heating apparatus, or a reflux apparatus\textsuperscript{18, 20-21}. Alternate pathways notably include light-based irradiation, which has been shown to create similar species\textsuperscript{7}. In this work we demonstrate an alternative novel synthetic pathway for cage-like POVs that yields variable and distinct oxidation state compositions.
compared to the traditional hydrothermal techniques or irradiation techniques. Additionally, we demonstrate the utility of high-resolution mass spectrometry as a tool for the speciation of these POV nanocages down to the degree of oxidation.

**B: Methods**

Bulk electrolysis was carried out on a Pine WaveNow potentiostat, with an Ag/AgCl (3M KCl) reference electrode, a platinum counter electrode, and a reticulated carbon working electrode. $V_{15}O_{36}Cl$ synthesis was carried out by preparing a 100 mM solution of ammonium metavanadate (Sigma Aldrich) which was adjusted to pH 4.5 using hydrochloric acid. A potential of -0.3 V (vs Ag/AgCl 3M KCl) was applied for 24 hours. The product was then precipitated from solution by addition of ethanol and dried. Mass spectra were collected on a Solarix 7T FT-ICR-MS from Bruker Daltonics (Billerica, MA). Typically instruments parameters were capillary voltage of 4000 V, spray shield: -500 V, drying gas temperature: 150°C, data acquisition size: 1M, ion accumulation time: 0.245 seconds, and Q1 mass: 100 m/z.

Isotopic exchange experiments were conducted by first saturating $V_{15}O_{36}Cl$ with $^{18}$O by preparing 4 mg/ml solution of $V_{15}O_{36}Cl$ in H$_2^{18}$O (>97% purity, Cambridge Isotopes Labs, Cambridge, MA). The solution was then diluted 100-fold into a solution of 5 mM ammonium acetate adjusted to pH 7 using ammonium hydroxide, and MS measurements were subsequently taken at the described timepoints.
The reduction of V10 via bulk electrolysis resulted in the formation of V$_{19}O_n$(44 ≤ n ≤ 49), a V18 based POV cage encapsulating a metavanadate species (henceforth referred to as V19)$^{2,22}$. Mass spectra of this sample generated over the course of the subsequent synthesis of V$_{15}$O$_{36}$Cl (figure 4.1, reflective of the product from the original synthesis of V19 figure 4.2) demonstrated an array of mixed valence states, as well as significant heterogeneity in the number of oxygen atoms. Notably there is also a bimodal distribution that can be observed in the larger V19 molecules with 46 or more oxygen atoms and is more clearly resolved for larger species. The more reduced (higher m/z) population is constantly centered on 11-12 V$^{IV}$ centers, whereas the more oxidized population becomes more reduced on average as the number of oxygens decrease. This shift in the center of the oxidized population is the apparent cause of the loss of resolution, causing

![Mass spectrum](image)
the two populations to merge as the overall number of oxygens decrease. Previous synthesis of V19 POV cages led to two distinct confirmations, the first being a more condensed \( V^{IV}_{16}V^{V}_2O_{43}(VO_4)^{13^-} \) structure. The second is a larger and more oxidized \( V^{IV}_6V^{V}_{13}O_{49}^9^- \) generated in the presence of n-butylammonium\(^\text{22}\). While the mass spectrum shows two populations with the same/similar empirical formula, the valence states for the two populations are reversed from their literature counterparts, with the larger species containing on average 11-12 \( V^{IV} \) centers and the smaller containing from 5-10 on average.

Analysis of the bimodal distribution is further complicated when you look at the ratio of \( V^{IV} \) to \( V^{V} \) across all observed V\(_{19}\) species which shows a clear gaussian distribution, with an average...
of 9 V\textsuperscript{IV} centers. It is likely that many of the smaller V19 species are loss-of-water fragments like those observed for V10. Computational modeling predicts that O-H bonds weaken the bond of an oxygen with its corresponding vanadium centers, generating the loss-of-water fragments\textsuperscript{1, 23}. The higher proportion of V\textsuperscript{IV} centers also increases the number of hydrogen atoms observed in the gas phase which makes V19 more susceptible to this fragmentation pathway. The leftmost of the bimodal distributions typically only possesses at most one hydrogen indicating that it may be the terminal product of loss-of-water fragmentation. In this case the bimodal distribution comes from depletion of the species that would occupy that region, which instead fragment further. Because of this we conclude that the primary products of this synthesis are the larger V\textsubscript{19}O\textsubscript{n}(44 \leq n \leq 49) species, which can be observed in the right-most distribution. These species bear a close resemblance to V\textsuperscript{IV}\textsubscript{12}V\textsuperscript{V}V\textsubscript{50}\textsuperscript{17} which was synthesized as an intermediate in the generation of an azide centered POV cage V\textsuperscript{IV}V\textsubscript{10}O\textsubscript{44}(N\textsubscript{3})\textsuperscript{7}. This method uses oxidative stress (as evidenced by the decrease in the relative amount of V\textsuperscript{IV}) in the final product, to cause the spontaneous encapsulation of azide\textsuperscript{16}. The analysis of the relative amount of V\textsuperscript{IV} present also describes the overall electric potential of the structure. Previous studies have shown that the additional
electrons resulting from the reduction of a $V^V$ center to $V^{IV}$ are delocalized, and affect the electric potential of the surface as a whole rather than any singular region\textsuperscript{24}. The same study also demonstrated that the cage will be reduced preferentially over the payload, providing some degree of protection.

The mass spectra of V19 gives a strong indication that it serves as an intermediate in a bulk electrolysis based POV cage synthesis. The optimized set of synthetic parameters described previously was used to drive the reaction to completion. These reaction conditions yielded the distinct product $V_{15}O_{36}Cl$, a chloride centered POV (figure 4.4) as well as

\textbf{Figure 4.4.} Negative ion mode mass spectrum of $V_{15}O_{36}Cl$ from a solid sample reconstituted in water at a concentration of approximately 1 mg/ml. Insert shows isotopic distribution of $H_2V^{IV}_{7}V^{V}_{8}O_{36}Cl^{3-}$ and related species. Relevant populations are labeled by the identity of their most abundant peak.
some V19 impurities (figure 4.1). Small quantities of $V_{22}O_{55}^{7-}$ were also observed, which resemble another previously synthesized POV$^{25-26}$. Notably the chloride centered structure differs from the V19 structure in that the payload is no longer bound to oxometallate framework and instead is free floating within the cage structure (figure 4.3)$^4$. The chloride centered cage is also significantly more stable than the V19 variant, with strong $V_{15}O_{36}Cl$ signal observable after one week of storage in solution under ambient conditions whereas the V19 signal has degraded in its entirety (figure 4.5). The MS data indicates that the average ratio of $V^{IV} : V^{V}$ is 8:7. Chloride centered POV cages
have been reported previously with similar ratios of $V^{IV}$:$V^{V}$ although they were predominantly synthesized hydrothermally rather than electrochemically\(^4\).\(^{27}\).

MS/MS measurements were used to confirm that the chloride ion observed in the MS results was located within the cage structure (figure 4.6). It was observed that with mild collisional activation loss of O, HO$_2$, HClO and HClO$_2$ could be observed, with no loss of chlorine by itself. This indicates the chloride ion is localized within the cage structure as some loss of oxygen is necessary for its release. The loss of oxygen likely compromises the cage structure such that the chloride ion can effectively escape alongside oxygen, although this leaves some ambiguity as to whether it escapes as a chloride ion or as HClO or HClO$_2$. While HV$_{15}$O$_{36}$Cl$_2^-$ is the most abundant parent ion, more reduced species that were co-isolated also contributed to the fragments observed. It is apparent that the species which allowed for the chloride ion to escape were largely comprised of $H_3V^{IV}_5V^{V}_8O_{36}^{2-}$ (seen in the loss of HClO$_2$) and to a lesser degree $H_3V^{IV}_7V^{V}_8O_{36}^{2-}$ (loss of HClO). The

Figure 4.6. Negative ion mode msms analysis of HV$_{15}$O$_{36}$Cl$_2^-$ with an isolation window of 8 m/z and CID voltage of 30 V. Parent ion is labeled with formula, and fragments are labeled with lost atoms.
higher abundance of \( \text{H}_5\text{V}^{\text{IV}}\text{V}_5^{\text{V}}\text{O}_{36}^{2-} \) indicates that more reduced species preferentially lose the chloride payload. This behavior indicates either a reduced affinity for the anionic payload due to the more electron rich nanocage, or that the increased number of electrons destabilizes the cage making it more susceptible to gas-phase fragmentation.

To further probe the solution phase dynamics of the chloride centered nanocage, oxygen 18 isotopic exchange was employed in the hopes that it could help elucidate novel metastable states as it did for V10. V15 with its unique cage like structure and its higher proportion of V\(^{\text{IV}}\) centers makes it a notable contrast to V10's structure and performing O18 exchange on it would reveal if these led to any differences in the dynamics. O18 exchange analysis was performed as described in chapter 3, save for the pH of the final solution which was adjusted to 7 using ammonium hydroxide.

The resulting exchange profile shows several significant differences from those observed for V10 (figure 4.7). While the overall exchange process occurs on a similar timescale to that of V10 in pH 4 under ambient conditions, the way the exchange reaches completion differs drastically. Many more distinct populations were observed for the exchange of V15, although again there was a relatively slow uncorrelated exchange style peak shifting rightward at first. The subsequent populations are significantly narrower than those observed for V10, and the peak centers are mostly stationary across later timepoints. Instead, the relative abundance of each population changes until an endpoint is reached.

This unique exchange profile indicates that the slow exchange of surface oxygen for the static structure plays a much less significant role in this exchange profile than it did for V10. The
Initial uncorrelated exchange likely represents most of the exchange of the outward facing terminal oxygen bound to each V center. From this point on the exchange is dominated by a stepwise exchange profile made up of well-defined shifts in O16 incorporation which could be indicative of two potential exchange mechanisms. The first is a scenario in which a metastable state is sampled that transiently exposes a small region of the POV cage (Figure 4.8.a) creating four
vulnerable oxygens. This exposed subunit then rapidly exchanges to completion before being folded back into the cage structure. This would explain the roughly three oxygen spacing of the observed peaks in the exchange profile. The other potential explanation is that the exchange profiles represent the incorporation of whole metavanadate subunits (figure 4.8.b). At neutral pH some proportion of vanadate in solution will be metavanadate, which has been shown to exchange oxygen rapidly in solution\(^28\). When one metavanadate unit from solution replaces a subunit in the POV cage, it would exchange at most three oxygens (assuming the terminal oxygen has already been exchanged), which matches the shift seen in the exchange profile. Both these mechanisms would conform to correlated exchange dynamics wherein the rate of exchange significantly outpaces the rate of the unfolding/dissociative event\(^29\). Either of these two mechanisms would be a significant departure from that observed for V10.

An important note is the implication both these mechanisms have for the stability of the payload. Both processes would potentially generate an opening of a sufficient size for the free-floating chloride center (and other payloads if these same events are observed in other nanocages) to escape and be substituted with another molecule in solution. Potential exposure of
the payload to the environment would be an important attribute to watch out for in potential use cases involving fragile/volatile payloads, such as the sensing of nitric oxide\textsuperscript{10}.

**D: Conclusion**

The nanocages generated in this work demonstrate that bulk electrolysis is capable of synthesizing POV cage structures previously reported to have been synthesized hydrothermally. This process is tunable, as the payload of the resulting cage can be controlled, and the duration for which the reduction potential is applied can determine the final product and likely plays a role in determining the relative amount of V\textsuperscript{IV}. The tunable nature allows for the generation of V19 cages used a precursor of other products, packaging volatile payloads that may be otherwise too difficult to work with. Additionally, the V19 species generated via bulk electrolysis have unique V\textsuperscript{IV}:V\textsuperscript{V} ratios which could lead to unique reactivity.

Bulk electrolysis can also replicate cage structures like V\textsubscript{15}O\textsubscript{36}Cl without the higher temperature and pressure that may be necessitated by hydrothermal synthesis allowing for the potential synthesis of cage structures with temperature sensitive payloads. Analysis of these cage-like POVs also demonstrates that mass spectrometry is an invaluable technique for their characterization. It can rapidly speciate them both by mass and by distribution of oxidation states which can reveal unique populations with different average levels of V\textsuperscript{IV}. Furthermore, oxygen exchange analysis can be used to reveal the existence of metastable states that could affect the stability of the payload or otherwise compromise the nanocage structure.
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CONCLUSION

In this work we have shown that mass spectrometry is a valuable technique for the analysis and speciation of POMs. Initial analysis of V10 using high resolution mass spectrometry in chapter I demonstrates the utility of mass spectrometry in characterizing POMs and confirming theorized properties. MS/MS analysis of V10 revealed loss of water fragments generated in the gas phase, which were indicative of the effect that hydrogen bound at oxometallate surfaces has on the bonds between oxygen atoms and metal centers.\(^1\) High resolution MS of V10 also revealed the existence of partially reduced V10 species containing one or more V\(^{IV}\) centers. LC-MS performed using ion exclusion chromatography was able to confirm the solution phase origin of these reduced species. The ability of HRMS to resolve and identify different compositions of oxidation states is particularly important to catalytic and electronic work involving POMs which relies heavily on the ability of POM complexes to accept electrons (through reduction of metal centers).\(^2\)\(^-\)\(^3\) As mentioned in chapter II, many of the biological interactions of POMs are electrostatically driven. The reduction of POMs can result in a delocalized change in electron density, such that even a single reduction event can impact the electric potential of the whole POM.\(^4\) Complete speciation of POMs including by oxidation state distribution is thus also important in studying their biological interactions. The initial work performed demonstrates the ability of MS analysis to identify POM species by their composition of oxidation states, and its ability to assess the stability of POMs using MS/MS analysis.

In chapter II of this work native mass spectrometry was employed to investigate the ability of V10 to disrupt the SARS-CoV-2 infectivity cycle in a manner analogous to heparan sulfate.\(^5\)\(^-\)\(^6\) Peak fitting was used to deconvolute MS data of an RBD-V10 complex indicating that V10 does have affinity for regions of the spike protein. Subsequent analysis of the RBD-ACE2 complex revealed that V10 can disrupt the ability of the spike protein to bind to the ACE2 receptor at the
cell surface. The mechanisms of action that were identified via MS analysis were confirmed to be effective using cell viability studies although any therapeutic effect is overshadowed by the onset of the cytotoxic effects of V10. The ability of MS analysis to identify the mechanisms of action of POMs in biological systems, and to even localize these interactions to specific regions of proteins, shows how it could be used to better understand the role POMs play in biological systems. It can help in instances where the efficacy of a POM has been demonstrated but the mechanism by which it acts is unknown such as in anticancer applications that have been proposed or demonstrated.7

HRMS analysis coupled with oxygen exchange was employed in chapter III to character POMs and subsequently revealed transient dynamic events akin to the metastable states of proteins that can be observed in HDX. The exchange profiles of V10 reveal metastable states that can emerge in solution and lead to larger dissociative events that dramatically accelerate the exchange process. Peak fitting can translate these exchange profiles into kinetic information, describing the nature of the metastable states and the role they play in the dynamics of V10 in solution. Additionally, this technique theoretically only grows more capable as the size and complexity of POMs increases, because the higher the number of oxygens the higher effective resolution of the technique. The metastable states observed and the larger dissociative event they lead to has significant implications for applications of V10. In biomedical applications, smaller species are believed to have unique biological activity and increased cytotoxicity and as such controlling their emergence would be essentially to these applications.7-8 The structural instability revealed could also have implications for the catalytic activity of V10, and may play a role in determining optimal conditions for catalysis.9-10

The novel synthetic pathway for POV nanocages demonstrated in chapter four demonstrated the capability of mass spectrometry in distinguishing and confirming the identity of
newly synthesized POMs. MS analysis was able to identify V19 nanocages by both empirical formula and amount of reduced V\textsuperscript{V} centers, confirming that the new nanocage possessed a unique ratio of V\textsuperscript{V}:V\textsuperscript{V} centers. We were also able to confirm that this synthesis can be used to prepare the chloride centered V\textsubscript{15}O\textsubscript{36}Cl with V19 serving as a precursor. The solution phase dynamics of the chloride centered nanocage were then probed using oxygen exchange analysis revealing an exchange profile that departs significantly from that seen for V10. The analysis of the POV nanocages demonstrates how MS can be used to analyze unknown POMs, separating them by species both by formula and by oxidation state composition. Further analysis can then be done to look at the dynamics of the nanocage in solution using oxygen exchange. The ability to detect low abundance and transient POV species sets MS analysis apart, able to detect potential contaminants or instabilities in POM complexes that may interfere with their applications. Alternatively, the low abundances species or unstable conformations may be the actual POM species interacting with the system as was postulated for anticancer applications.\textsuperscript{7}

The analysis of the fundamental qualities of POMs presented in this work describes a significantly more complicated set of solution phase properties than was previously conceived. Initial analysis of V10 revealed that partially reduced species exist naturally in solution, and subsequent oxygen exchange analysis revealed transiently sampled metastable states of V10 exist and can lead to large scale dissociative events. Beyond the implications of this work for applications of V10, the fact that POMs can possess these attributes implies that more detailed analysis of POMs may be necessary to fully understand the role they play in potential applications.

Going forward there remain several avenues of POM analysis to explore. For V10 the oxygen exchange analysis raised questions about the role partially reduced species of V10 play in its solution phase dynamics. Direct analysis of partially reduced V10 resulted in an exchange profile nearly identical to that of fully oxidized V10. Indicating that the rate of interconversion
between reduced and fully oxidized forms of V10 is high enough that any differences between the two states is averaged out. Attempts to reduce V10 resulted in the synthesis of V19 nanocages, but a method capable of enriching the level of partially reduced V10 is still possible with sufficient tuning. Repeating the oxygen exchange study with a higher level of partially reduced species would identify any differences observed as coinciding with partially reduced species. If partially reduced species were shown to impact the dynamics of V10 in solution it would impact applications in which reduction of POMs is a common/inherent part of there use such as catalytic or electronic applications.2–3

A shortfall of oxygen exchange analysis coupled with HRMS is that it cannot localize the exchange to specific oxygens/regions of the POM complex. One method to supplement this analysis would be to perform NMR alongside MS (utilizing 17O instead of 18O) to determine which structurally unique oxygens are exchanged at each timepoint, and how this coincides with the sampling of metastable states. NMR would help to confirm or at least narrow down the potential metastable states. It would also serve as a powerful complimentary technique for the future analysis of POMs using oxygen exchange, localizing the results in the same manner peptide level analysis can localize HDX results. MS analysis overall may also have difficulty analyzing POMs containing transition metals with more complicated isotopic distributions like molybdates and tungsten containing species.

In chapters I-III we utilized and developed a wide variety of techniques for the analysis of POMs, which when combined yield a robust toolkit for their characterization. This culminates in chapter four in which we utilized this toolkit to characterize POV nanocages resulting from a novel synthetic pathway. The utility of the MS toolkit developed here has implications for the analysis of the fundamental characteristics of POMs as well as their applications in the fields of catalysis, electronics, and biomedicine. When combined with the solution phase properties of POMs
observed in this work, this toolkit and MS generally is likely to play an important role in POM work going forward. The capabilities of MS and its complimentary techniques, as evidenced in this work, make it an excellent choice for future studies of POMs although more work remains to be done in the development of techniques for POM analysis.
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