JACS Au
AN OPEN ACCESS JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Perspective

Exploring the Reactivity of Polyoxometalates toward Proteins: From Interactions to Mechanistic Insights

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Cite This: *JACS Au* 2023, 3, 978–990

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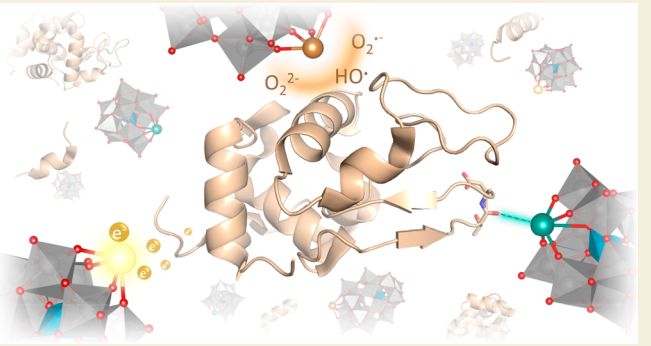
ABSTRACT: The latest advances in the study of the reactivity of metal-oxo clusters toward proteins showcase how fundamental insights obtained so far open new opportunities in biotechnology and medicine. In this Perspective, these studies are discussed through the lens of the reactivity of a family of soluble anionic metal-oxo nanoclusters known as polyoxometalates (POMs). POMs act as catalysts in a wide range of reactions with several different types of biomolecules and have promising therapeutic applications due to their antiviral, antibacterial, and antitumor activities. However, the lack of a detailed understanding of the mechanisms behind biochemically relevant reactions—particularly with complex biological systems such as proteins—still hinders further developments. Hence, in this Perspective, special attention is given to reactions of POMs with peptides and proteins showcasing a molecular-level understanding of the reaction mechanism. In doing so, we aim to highlight both existing limitations and promising directions of future research on the reactivity of metal-oxo clusters toward proteins and beyond.



KEYWORDS: *Metal-oxo clusters, Polyoxometalates, Biomolecules, Proteins, Proteomics, Hydrolysis, Redox reactions*

1. INTRODUCTION

The natural presence of metallic clusters in biological systems is an endless source of chemical lessons, and inspiration for a vast array of scientific fields. The diversity of biomolecules and metallic clusters in terms of nature, size, shape, charge, composition, solution behavior, and surface chemistry renders these bioinorganic systems with a unique and fascinating complexity. Moreover, these hybrid systems serve important biological functions in living organisms, playing a key role in vital biochemical transformations, typically in combination with proteins. Clear examples of this are the $CaMn_4O_5$ metal-oxo core of photosystem II, which catalyzes water-splitting during photosynthesis in certain organisms, and iron−sulfur clusters, which act as protein cofactors in many biochemical processes.[1,2] Hence, research areas addressing the synergy between metallic clusters and proteins are rapidly growing due to their potential in the development of new types of drugs,[3,4] sensors,[5,6] artificial enzymes,[7,8] and novel biomaterials.[9,10]

Metal-oxo clusters in particular are one of the most diverse and versatile groups among the many families of metallic clusters.[11] These clusters can be anionic or cationic in nature. However, to date, research on the reactivity of metal-oxo clusters toward biomolecules has mainly focused on anionic polyoxometalate clusters (POMs). While cationic clusters often need to be stabilized by organic capping ligands, rendering their overall structure neutral, anionic POM clusters are typically stable without any ligands.[12] Although reports on the chemistry of cationic or neutral metal-oxo clusters toward biomolecules are emerging,[13] especially in the context of porous metal−organic frameworks (MOFs),[14] POMs have been more extensively employed in areas such as crystallization assays,[15] bioactivity studies,[4,16−18] and the development of artificial enzymes.[8,19] In addition, POMs stand out over other discrete inorganic clusters because many POM structures with varying elemental composition, overall geometry, solution behavior and diverse physicochemical properties are readily available from inexpensive inorganic salts.[20,21] Moreover, their chemical properties can be rationally designed as needed through varying their size, shape, charge, composition and counterion. Therefore, POMs provide a strong starting point

Received: January 5, 2023
Revised: January 31, 2023
Accepted: February 1, 2023
Published: February 13, 2023

https://doi.org/10.1021/jacsau.3c00011
JACS Au 2023, 3, 978−990

for investigating the chemistry between inorganic metal-oxo clusters and biomolecules.

Despite the promising properties of metal-oxo clusters, the lack of a clear molecular-level understanding regarding their interactions and reactivity toward biomolecules hampers further developments in the rational design of such clusters for applications in biochemistry and medicine. This can be clearly seen from the enduring unanswered questions on the mechanisms of action of POMs that give rise to their reported antiviral, antibacterial, and antitumor activity.[4,16,19] Nevertheless, the therapeutic activity of POMs has been largely attributed to their interactions and reactivity toward proteins.[4,19] Hence, elucidating the molecular aspects of the reactivity between well-defined, discrete metal-oxo clusters and proteins would offer invaluable insights to advance, for instance, essential analytical tools across the biomedical sciences,[22,23] or the development of novel types of inorganic drugs based on metal-oxo clusters.[4] This challenge can be addressed through the judicious study of simpler systems, such as peptides, which can be very instructive by unveiling a deeper understanding that can be built up toward larger and more complex biological systems in a "bottom-up" approach. Therefore, to prompt research in this underexplored area of bioinorganic chemistry, we discuss here the main types of biochemical transformations of proteins and peptides that can be catalyzed by POMs. These include hydrolysis reactions, which are promoted by the Lewis acidic properties of POMs, and redox reactions, which occur due to the ability of POMs to reversibly donate and accept electrons. Moreover, particular emphasis is given to understanding the driving forces behind the observed interactions and reactivity. With this perspective in mind, we outline here the inspiring lessons learned from interaction and reactivity studies between anionic metal-oxo clusters and proteins. These lessons may enlighten pivotal future research ventures exploring the enormous potential of metallic clusters in biology, medicine, and related areas.

2. POM–PROTEIN INTERACTIONS

The reactivity of POMs toward proteins is highly dependent on how they interact with each other in solution, especially in terms of the selectivity of the reaction. Therefore, understanding these interactions is essential for developing and optimizing POMs as agents that can promote important chemical transformations of proteins. Furthermore, it is necessary to investigate these interactions to gain detailed insights into the biological activity of POMs and their mechanism of action on a molecular level.

The nature of POM-protein interactions is intrinsically linked to the structure and composition of POMs. The majority of commonly investigated POM compounds are based on the four most common archetypical structures, (**1**) Lindqvist ($[A_6O_{19}]^{n-}$), (**2**) Anderson–Evans ($[XA_6O_{24}]^{n-}$), (**3**) Keggin ($[XA_{12}O_{40}]^{n-}$), and (**4**) Wells–Dawson ($[X_2A_{18}O_{62}]^{n-}$), which are composed of $\{AO_6\}$ octahedra (where A is typically Mo^{6+}, W^{6+} or V^{6+}) and may contain templating heteroatoms (X = P^{5+}, Si^{4+}, Mn^{3+}, etc.) as shown in Figure 1. Furthermore, these structures can be systematically tuned to influence POM-protein interactions, which are generally governed by several factors, including the charge, size, shape and composition of the POM.[24−26]

Due to their anionic and oxygen-rich nature, POMs predominantly form electrostatic interactions with proteins. As evidenced by numerous reported crystal structures,[15,27−29]

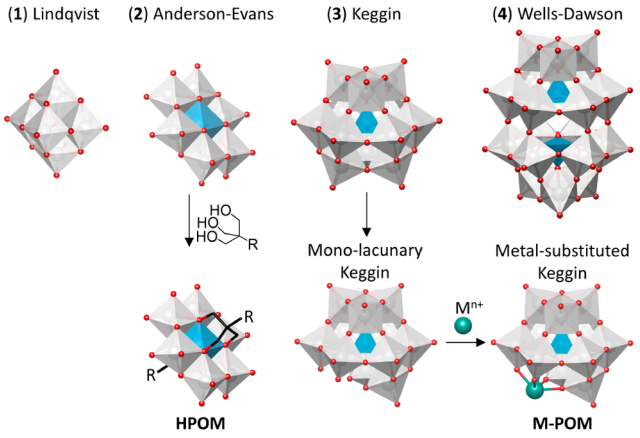


Figure 1. Structures of the most common types of polyoxometalates: (**1**) Lindqvist ($[A_6O_{19}]^{n-}$), (**2**) Anderson–Evans ($[XA_6O_{24}]^{n-}$), (**3**) Keggin ($[XA_{12}O_{40}]^{n-}$), and (**4**) Wells–Dawson ($[X_2A_{18}O_{62}]^{n-}$) as well as representative examples of the synthetic approach toward metal-substituted POM complexes (M-POMs) and hybrid organic–inorganic POM structures (HPOMs). $\{AO_6\}$ octahedra in gray composed of oxygen in red and metal centers in gray; $\{XO_6\}$ octahedra and $\{XO_4\}$ tetrahedra in blue; carbon chain in black; metal ion in teal.

POMs tend to favor interactions with regions of proteins containing positively charged functional groups, such as the ammonium and guanidinium groups of lysine and arginine amino acid residues, respectively (Figure 2a).[27,30] In addition, crystal structures have also shown that various hydrogen bonding close contacts can form between bridging or terminal oxygens of the POM and polar uncharged amino acid residues (e.g., tyrosine, serine, and asparagine) on the surface of proteins, either directly or mediated by water or metal ions (Figure 2a).[27,31] These charge–charge and hydrogen bonding interactions between POMs and proteins have also been confirmed by molecular dynamics simulations, isothermal titration calorimetry (ITC) and several spectroscopic methods.[24,30,32,33] Hence, electrostatic interactions between POMs and proteins have been established to be the main driving forces behind their binding, both in solution and the solid state.

Along with the charge, the interplay between the size and shape of POMs with that of proteins is also a determining factor in the strength of their interactions since proteins can vary significantly in their three-dimensional shapes and have a heterogeneous distribution of surface charges. The ability of POMs to interact with proteins through multiple amino acid residues at once, due to the generally large size of these clusters, can result in good binding affinities arising from cooperative effects.[30,34] However, in order to maximize these binding interactions there needs to be good complementarity between the shape and size of the POM and the interacting surface of the protein. For instance, a computational study in which the size and shape of the POM was varied systematically while the charge density was kept constant, found that a Keggin-type POM had the highest affinity for lysozyme since it displayed the best fit with the cavities on the surface of the protein.[33] In contrast, a smaller Lindqvist-type POM displayed less persistent interactions with lysozyme, due to its inability to interact with multiple amino acid residues at once. Interestingly, larger POMs (e.g., Wells–Dawson) did not give rise to more favorable interactions, as the size of the

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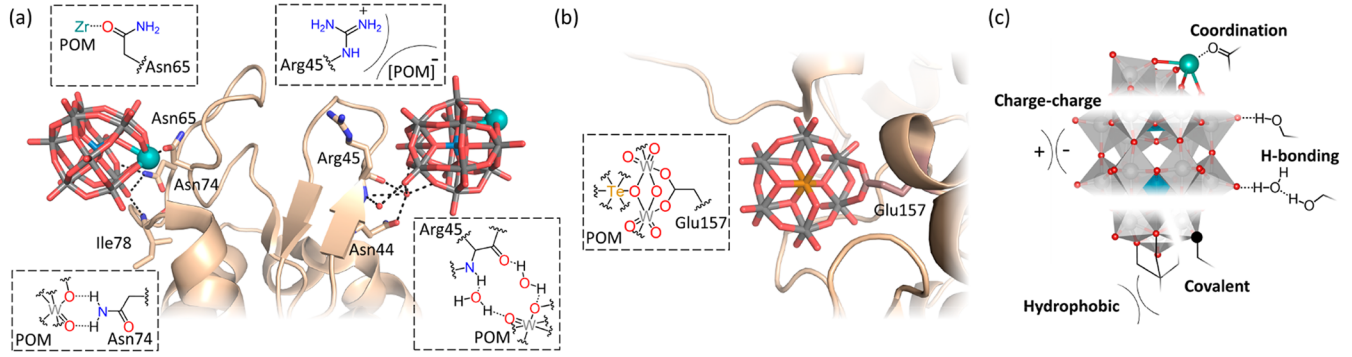

Figure 2. (a) Crystal structure of $[Zr(PW_{11}O_{39})]^{3-}$ with lysozyme where the POM interacts with the protein via charge−charge and (direct or water-mediated) hydrogen bonding interactions as well as through coordination of a carbonyl group to Zr^{4+} (PDB code 6GNL). (b) Crystal structure of $[TeW_6O_{24}]^{6-}$ covalently bound to a protein through the carboxylate group of a glutamate residue (PDB code 4Z13). Skeletal structures depicting the interactions have been included in insets alongside the crystal structures for clarity. (c) Illustrative general representation of the different types of binding interactions possible between POMs and proteins.

protein cavities limited the interactions to only a small part of the POM structure.[33] This is in agreement with experimental results on the binding interactions of different POMs with a designer protein (Tako8), which showed that the larger Keggin and Wells−Dawson POMs displayed stronger interactions compared to smaller POMs, as they could fit better within the large positively charged cavities of the protein.[35] Furthermore, the structure of a protein can limit or favor reactivity, and in some cases strongly interacting POMs have been shown to induce conformational changes in the structure of the protein, such as partial unfolding resulting in loss of α-helical and/or β-sheet content.[26,36,37] Hence, both the structure of the POM and that of the protein need to be considered when investigating their interactions and the ensuing reactivity.

The influence of the charge and size of POMs on their interactions with proteins also needs to be considered together, since the charge density of POMs plays a key role. As the charge density increases, the strength of the POM-protein electrostatic interactions increases. However, the energy cost of POM desolvation also becomes higher as the charge density increases, which makes protein binding less favorable for POMs with high charge densities despite the stronger electrostatic interactions. Therefore, due to the need for a balance between these two opposing factors, POMs with intermediate charge densities are more likely to have higher binding affinities.[33,38] In addition, POMs with relatively low charge densities display chaotropic behavior (i.e., they disrupt the hydrogen bonding network formed by water molecules).[33,39] This means that they are weakly hydrated, and are more likely to interact with hydrophobic and neutral polar organic moieties due to a lower entropic penalty of desolvation. The influence of such chaotropic effects on POM-biomolecule interactions has been particularly investigated with cyclodextrin, a macrocyclic carbohydrate that can form host−guest interactions with POMs.[40,41] However, chaotropic effects have not been extensively explored experimentally with proteins or other biomolecules, especially in the context of their reactivity.

The binding strength and stoichiometry of the interactions between POMs and proteins has also been shown to depend on the solution conditions, such as the ionic strength, pH, and temperature. Increasing the ionic strength of the solution was reported to decrease the binding affinity of POMs toward proteins, due to shielding of the electrostatic interactions.[24,25,42] In contrast, the influence of the temperature and the pH on POM-protein interactions displayed less clear trends, which is likely linked to how these factors affect the structure and the surface charge of the protein.[25,42] This can be seen from the increase in binding interactions of $[Ce(PW_{11}O_{39})_2]^{10-}$ with lysozyme when increasing the temperature from 25 to 37 °C, while increasing the temperature from 25 to 45 °C was reported to "switch off" the interactions between $[EuW_{10}O_{36}]^{9-}$ and another protein, human serum albumin (HSA).[25,43] Similar scenarios have been reported when the pH was varied, since the net surface charge of a protein, typically represented by its isoelectric point (pI), depends on the pH and its structural conformation can also be affected by the pH.[25,42,44] For example, lowering the pH from 7.4 to 4.4 caused a decrease in binding stoichiometry of $[Ce(PW_{11}O_{39})_2]^{10-}$ to lysozyme (pI = 11),[25] while the binding stoichiometry of $[H_2W_{12}O_{40}]^{6-}$ to HSA (pI = 5.9) increased upon lowering the pH.[42] This contrasting behavior is likely due to differences in the structural properties of the proteins. Nevertheless, in both cases higher binding affinities were observed at lower pH, due to stronger attractive electrostatic interactions.[25,42] Therefore, it is important to always take into account the impact of the reaction conditions when evaluating POM-protein interactions since the ionic strength, temperature and pH can have a significant effect on these interactions.

Along with the shape, size, and charge of POMs, the composition of POMs can be altered through well-established synthetic steps to give rise to modified POM structures that typically fall within one of two subclasses of POMs: metal-substituted POM complexes (M-POMs) and hybrid organic−inorganic POM structures (HPOMs). M-POMs are generally prepared through the controlled removal of one or more metal centers of the POM structure and their corresponding oxo ligands followed by complexation of metal cations (Figure 1).[45−47] On the other hand, HPOMs can be synthesized through several strategies that result in the covalent attachment of organic ligands onto the surface of the POM (Figure 1).[48,49] These modified POMs represent particularly versatile platforms for developing multifunctional structures of varied potential utility, which can be exploited in biological systems.

The structural versatility of POMs toward modifications of their structure and composition can be leveraged to enable additional interactions with proteins. Coordination of the substituted metal center in M-POMs to nitrogen or oxygen

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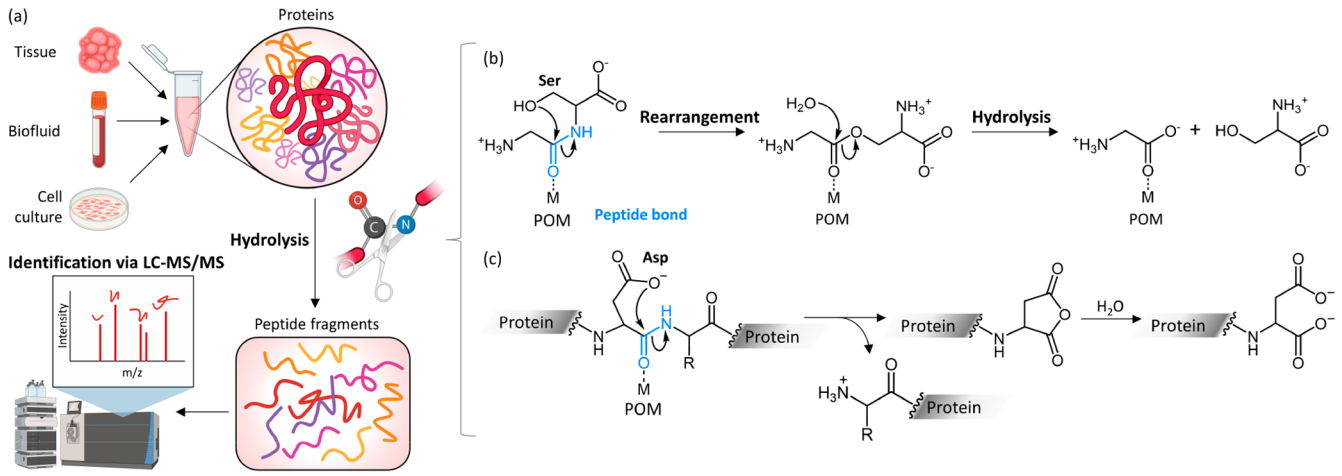

Figure 3. Scheme depicting (a) the general strategy for the characterization of proteins via LC MS/MS on hydrolytic fragments. General reaction mechanisms for the POM catalyzed hydrolysis of (b) a glycine-serine dipeptide (X-Ser) via a rearrangement and of (c) a protein at peptide bonds downstream of aspartate residues (Asp-X). Created with Biorender.com.

atoms (Figure 2) in proteins (e.g., side chain of histidine, peptide carbonyl group, etc.) has been reported to result in enhanced and more specific interactions that can play an important role in the reactivity of POMs.[36,50,51] In addition, covalently grafting organic moieties onto POMs increases the number of possible interactions and the specificity that can be achieved, particularly through hydrophobic interactions that cannot take place for all-inorganic POMs.[52−54] For example, the functionalization of an Anderson−Evans HPOM with aromatic moieties was shown to give rise to additional hydrophobic and $\pi-\pi$ interactions, resulting in stronger binding interactions with a serum albumin protein compared to the corresponding all-inorganic POM.[55] Recently, we have also shown that the strength and selectivity of the binding interactions of Anderson-Evans HPOMs with lysozyme strongly depend on the functional groups attached to the POM core.[44] It was observed that a more complex ligand bearing more functional groups capable of interacting with multiple protein residues resulted in stronger binding interactions, and that hydrophobic interactions were also likely responsible for enhancing the overall binding affinity. Moreover, the binding interactions of the HPOMs were significantly greater compared to the corresponding organic ligands or the all-inorganic POM on their own.[44] This shows that stronger interactions can be achieved through synergistic contributions of the organic and inorganic components. Nevertheless, despite these promising seminal results, the vast potential of HPOMs and their diverse functionality to drive POM-protein interactions in biological systems remains largely underexplored. Moreover, it has also been demonstrated that POMs can be covalently attached directly onto peptides and proteins (Figure 2b).[56,57] However, compared to supramolecular interactions, covalent binding has been explored much less in the context of reactivity.

Overall, through a combination of X-ray crystallography,[15,27] computational modeling,[58] ITC,[24,25] and several spectroscopic methods,[26,42,59,60] it has been shown that multiple types of interactions are possible between POMs and proteins: charge−charge, hydrogen bonding and van der Waals noncovalent interactions, as well as coordinative and covalent binding (Figure 2c).[15,61] Hence, current studies already provide important insights about how POMs interact with proteins in general, which allows for a mechanistic understanding of the reactivity of POMs toward proteins.[19,62] However, since multiple factors (charge, size, shape, composition, and solution conditions) have an influence on POM−protein interactions, clear trends on the impact of some factors are still lacking. Furthermore, the promising potential of HPOMs and their diverse functionality remains to be explored further.

3. HYDROLYTIC ACTIVITY OF POMs

Proteins are macromolecules composed of amino acids linked via peptide bonds that play an essential role in many biological processes. As a result, the hydrolysis of peptide bonds resulting in protein fragments is important for a wide range of applications in the food, leather, and detergent industries as well as in biochemistry, biotechnology, pharmacology and medicine.[63] The fragmentation of proteins via peptide bond hydrolysis is also key for the field of proteomics, that is, the systematic study of the structure, function, expression, modification, localization, interaction, and cellular activity of proteins.[63−65] The analysis of protein structures in proteomics typically involves the extraction, isolation and controlled hydrolysis of a particular protein into smaller, well-defined peptide fragments that can be more easily characterized by liquid chromatography tandem mass spectrometry (LC MS/MS) than the intact protein (Figure 3a).[66] However, the natural enzymes or the alternative chemical agents currently available for protein fragmentation have several disadvantages such as producing mainly short peptide fragments or requiring harsh and toxic reaction conditions, respectively.[65,67−68] Therefore, the ability of POMs to act as artificial proteases that can promote the selective hydrolysis of peptide bonds in proteins has been extensively explored by our group in recent years.

3.1. Hydrolysis of Peptides and Proteins by Oxometalates

Initial studies explored the hydrolytic activity of oxovanadates and oxomolybdates in solution toward different dipeptides as model substrates for proteins.[69,70] The reaction mechanism was determined to generally involve the coordination of the terminal amine group and the amide carbonyl oxygen atom to V^{5+} or Mo^{6+} metal centers, thereby activating the carbonyl group toward nucleophilic attack by water.[70] In addition, faster

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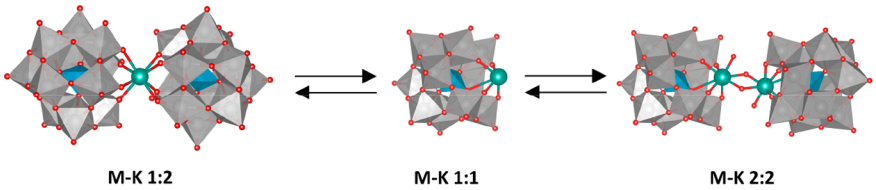

Figure 4. Equilibria between $[M(PW_{11}O_{39})_2]^{10-}$ (M-K 1:2), $[M(PW_{11}O_{39})]^{3-}$ (M-K 1:1), and $[\{M(\mu\text{-}OH)(H_2O)(PW_{11}O_{39})\}_2]^{8-}$ (M-K 2:2) in solution (M = Zr^{4+} or Hf^{4+}). Similar equilibria are also observed for $[M(P_2W_{17}O_{61})_2]^{16-}$ (M-WD 1:2), $[M(P_2W_{17}O_{61})]^{6-}$ (M-WD 1:1), and $[\{M(\mu\text{-}OH)(H_2O)(P_2W_{17}O_{61})\}_2]^{14-}$ (M-WD 2:2).

hydrolytic rates were systematically observed for dipeptides containing serine (X-Ser) due to the intramolecular attack of serine's side-chain −OH group toward the activated amide carbonyl, leading to a lactone intermediate that is prone to undergo a favorable *N,O*-acyl rearrangement (Figure 3b).

Based on the promising results obtained with dipeptides, the hydrolytic activity of oxomolybdate was also investigated toward the cleavage of peptide bonds in lysozyme.[71] Interestingly, cleavage selectively occurred at peptide bonds downstream of aspartate residues (Asp-X) due to the intramolecular nucleophilic attack of the −COOH group of the aspartate (Asp) side chain toward the amide carbonyl group activated by Mo^{6+} (Figure 3c).[71] However, this selectivity toward hydrolysis at Asp-X peptide bonds in the protein is in contrast to the preferred X-Ser hydrolysis observed in dipeptides and the reasons for this discrepancy are not yet fully understood. Hence, while simple model systems are useful for preliminary measurements to establish reactivity, this showcases the limitations of model systems to predict in some cases the reactivity in more complex biomolecules.

The catalytically active species in the hydrolysis of peptides and proteins by oxovanadates and oxomolybdates were proposed to be the monomeric orthovanadate ($[VO_4]^{3-}$) and orthomolybdate ($[MoO_4]^{2-}$). However, the highly dynamic behavior of vanadate and molybdate species in aqueous solutions makes it difficult to determine the exact nature of the catalytically active species, thereby complicating reaction optimization. As a result, further investigation of the hydrolytic activity of POMs shifted toward more stable tungstate M-POMs. Furthermore, these M-POMs can be tuned in terms of shape, size, and charge of the POM framework, as well as by changing the embedded metal ion, thus allowing for a systematic study of structure−activity relationships.

3.2. Hydrolysis of Peptides and Proteins by M-POMs

Different structural archetypes of tungstate M-POMs−such as the Lindqvist, Keggin, and Wells−Dawson−with embedded metal ions have been investigated for their reactivity toward peptides and proteins. These POM frameworks serve to coordinate and stabilize the embedded metal ions, which in some cases tend to form insoluble gels in aqueous solutions on their own.[72,73] Initial screening of different embedded metal ions showed that Hf^{4+}, Zr^{4+}, and Ce^{4+} displayed the best hydrolytic activity due to their high coordination number, oxophilicity, and high Lewis acidity.[73] Accordingly, Hf^{4+}, Zr^{4+}, and Ce^{4+} substituted M-POMs have been shown to selectively hydrolyze a wide range of proteins, regardless of their secondary structure, molecular weight, and pI.[8] Interestingly, POM-catalyzed protein hydrolysis produced peptide fragments that generally ranged between 5 and 15 kDa. These fragments are ideal in size for middle-down proteomics, which has emerged in recent years as one of the methods of choice in the structural characterization of proteins since it facilitates LC MS/MS analysis of protein digests.[72,74−76] Moreover, the efficiency of M-POMs toward hydrolyzing proteins was determined to depend on three main factors: (i) the electrostatic interactions between the M-POM and the protein, (ii) the nature of the embedded metal ion, as well as (iii) the speciation and stability of the M-POM under the reaction conditions (e.g., buffer, pH, etc.).

As discussed previously, the shape, size, and charge of a POM have an influence on its interactions with proteins. As a result, these factors have been observed to affect the efficiency of the hydrolysis of different proteins−namely myoglobin, HSA and transferrin−which was promoted by several Zr^{4+}-substituted Lindqvist, Keggin, and Wells−Dawson M-POMs.[72,74,77] The Wells−Dawson Zr^{4+} M-POMs were observed to have the highest hydrolytic efficiency in the hydrolysis of HSA and transferrin, which was proposed to be due to their stronger association constant and greater ability to unfold the protein structure as a result of their higher negative charge.[74,77,78] However, the Keggin M-POMs showed higher hydrolytic efficiency toward myoglobin, indicating that the size and shape of the M-POM can also have a significant effect.[26,72] In contrast, the shape, size and charge of the POM framework did not seem to affect the selectivity of the cleaved peptide bonds, since all Zr^{4+} M-POMs tested gave the same cleavage patterns.[72,74,77]

The electrostatic interactions between the anionic POM framework and positively charged regions of proteins have an impact on the efficiency of the hydrolysis reaction since they are the main driving force behind the observed hydrolytic activity of M-POMs.[74] These electrostatic interactions between the M-POMs and the protein surface enable hydrolysis by placing the embedded Lewis acid metal center in close proximity to peptide bonds in the protein. This facilitates the coordination of the embedded metal ion to the peptide carbonyl group, thereby making the carbon atom more electrophilic and susceptible toward nucleophilic attack (Figure 3).

The embedded metal ion not only plays a key role in activating the carbonyl group, but also has an impact on the selectivity of the hydrolysis reaction. Hf^{4+} and Zr^{4+}M-POMs have shown high selectivity toward hydrolyzing peptide bonds downstream of Asp residues (Asp-X) in all proteins investigated, which is distinct from the typical selectivity of natural proteases or other reported metal complexes.[72,79] As observed for oxomolybdates, this is also due to an intramolecular nucleophilic attack of the activated peptide carbonyl group by the carboxylic group of the Asp side chain (Figure 3c) based on density functional theory (DFT) calculations.[79] However, Ce^{4+} M-POMs have generally shown a different type

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of selectivity in comparison to the hydrolysis of the same protein by Hf^{4+} or Zr^{4+}M-POMs, with peptide bond cleavage being observed more often at sites not involving Asp residues.[25,75,77] Even though the reason behind this difference in selectivity is not yet well understood, it may be linked to the observed partial reduction of Ce^{4+} to Ce^{3+} throughout the reaction.[80,81] Therefore, further work remains to be done in order to elucidate the driving forces behind the selectivity induced by the embedded metal ion.

As can be seen from the proposed mechanism of action of M-POMs, a prerequisite for the hydrolysis of peptides by M-POMs is that the embedded metal ion must have available coordination sites. However, Hf^{4+}, Zr^{4+}, and Ce^{4+} M-POMs tend to form dimers in solution (M-POM 1:2 or M-POM 2:2) in which the embedded metal centers are partially or fully coordinatively saturated (Figure 4). Nevertheless, it has been shown by theoretical studies and single crystal X-ray diffraction (SC XRD) that these M-POM dimers are prone to dissociate in the vicinity of proteins, giving the catalytically active coordinatively unsaturated monomeric form: M-POM 1:1 (Figure 4).[27,29,51] As a result, the dimeric form is typically used as a catalyst precursor that dissociates into the catalytically active monomeric species in solution. The ease of dissociation depends on the solution conditions and on the structure and composition of the M-POM, with M-POMs that dissociate more easily displaying higher reactivity.[26,72] Furthermore, the speciation of the M-POM in solution depends on the pH, concentration, temperature, ionic strength and dielectric constant of the medium.[29,82−84] However, the reaction conditions do not seem to affect the selectivity of Hf^{4+} and Zr^{4+} M-POMs. On the other hand, the conditions do have a large influence on the efficiency of protein hydrolysis. For example, acetate buffer (pH 5.5) was determined to be more suitable than phosphate buffer (pH 7.4) since more acidic conditions favor hydrolysis, and the strong coordination affinity of the phosphate ions toward the embedded metal center hinders M-POM reactivity.[72,84] Therefore, careful choice of the reaction conditions is needed to maximize the hydrolytic efficiency. Nevertheless, all studies showed that the hydrolysis of peptide bonds in proteins could be performed under relatively mild conditions, demonstrating the suitability of M-POMs as alternatives to harsh chemical agents.

3.3. Reactivity of M-POMs toward Peptides and Proteins in Surfactant Solutions

Unlike natural proteases, which lose their activity in the presence of surfactants, it has been shown that M-POMs are still hydrolytically active in surfactant solutions.[85,86] This is highly important since surfactants can be used to probe the structure of proteins.[86] Additionally, surfactants are typically used to dissolve poorly soluble proteins, such as membrane proteins, that are often underrepresented in proteomics studies despite their important roles in many biological processes.[87] Therefore, the hydrolytic activity of M-POMs in the presence of surfactants has been investigated.

Initial studies focused on the interactions of M-POMs with different types of surfactants. These studies showed that cationic surfactants induced precipitation while neutral or anionic surfactants do not interact with M-POMs.[82,88] Interestingly, M-POMs were observed to interact with the positively charged ammonium groups of zwitterionic surfactants without inducing precipitation, resulting in M-POM incorporation into the micellar structures.[82,88] Moreover, it was

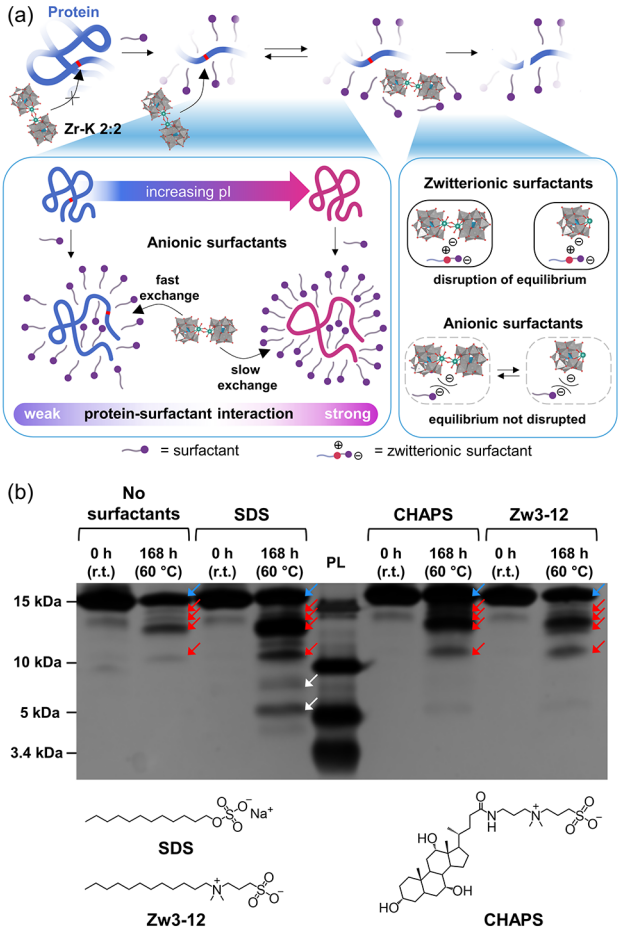

Figure 5. (a) Hydrolysis of a protein catalyzed by Zr-K 2:2 in the presence of surfactants, where the hydrolytic efficiency depends on the rate of exchange between the M-POM and the surfactant on the surface of the protein and on the influence of the surfactant on the speciation of the M-POM in solution. (b) Silver-stained SDS-PAGE gel showing the fragments formed from the hydrolysis of myoglobin immediately after mixing with Zr-K 2:2 and after incubation at 60 °C for 168 h in the absence and in the presence of SDS, CHAPS, or Zw3-12 (molecular weights determined using a protein ladder (PL) as reference). The blue and red arrows indicate the intact protein and the hydrolytic fragments, respectively, whereas the white arrows indicate the additional hydrolytic fragments observed in the presence of SDS. Adapted from ref 86. Copyright 2022 American Chemical Society.

shown that the hydrolytic activity of $[Zr(P_2W_{17}O_{61})_2]^{16-}$ (Zr-WD 1:2) toward the dipeptide glycyl-L-histidine was preserved in the presence of anionic, neutral, or zwitterionic surfactants as the surfactants did not affect the binding of the dipeptide to the M-POM.[88] Furthermore, in the presence of surfactants, M-POMs have also been reported to be hydrolytically active toward several globular and water-soluble proteins,[85,86,89,90] as well as toward unstructured and insoluble proteins.[87,91]

The hydrolytic activity of M-POMs toward proteins in the presence of surfactants has been determined to depend on: (i) the protein structure and pI, (ii) the structure of the surfactant (charge, size and polarity), (iii) the ease of exchange between the surfactant and the M-POM on the protein surface, as well as (iv) the influence of the surfactant on the speciation of the M-POM (Figure 5a).[86] The previously established ability of zwitterionic surfactants to interact with M-POMs can disrupt

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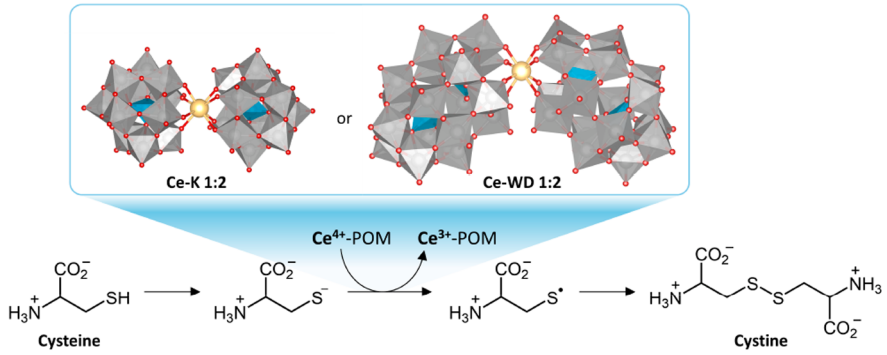

Figure 6. Radical homocoupling of cysteine (Cys) into cystine catalyzed by $[Ce(PW_{11}O_{39})_2]^{10-}$ (Ce-K 1:2) or $[Ce(\alpha_2\text{-}P_2W_{17}O_{61})_2]^{16-}$ (Ce-WD 1:2).

the equilibria between the dimer and monomeric forms of the M-POMs, which can negatively affect the hydrolytic activity (Figure 5a).[86] In addition, the hydrolytic reaction depends on the ability of the M-POM catalyst to exchange with the surfactant on the protein surface. Therefore, the strength of the surfactant-protein and POM-protein interactions, resulting from the interplay between the structure of the protein and that of the surfactant, have an influence on the efficiency, and even selectivity, of hydrolysis depending on the structure of the surfactant used and on the structure and pI of the protein (Figure 5). For example, although myoglobin is structurally similar to cytochrome c, additional cleavage sites and a higher hydrolytic efficiency were observed when myoglobin was hydrolyzed by an M-POM in the presence of the anionic surfactant SDS (Figure 5b).[86,89] This higher hydrolytic activity was proposed to be due to the easier exchange between the M-POM and the surfactant on the protein surface, since the electrostatic interactions between SDS and myoglobin (pI ≈ 7) are likely weaker than for cytochrome c with a higher pI of around 10 (Figure 5a).[86] The importance of the ease of exchange between M-POM and surfactant on the protein surface is also reflected in the decrease in hydrolytic efficiency of M-POMs upon increasing the concentration of the surfactant. Moreover, this effect of the surfactant concentration was observed across widely different classes of proteins: HSA (a globular soluble protein), β-casein (an unstructured partially soluble protein) and zein (an insoluble protein that requires surfactants to be dissolved).[85,87,91] However, under the right conditions, the addition of surfactants can even be exploited to achieve significantly higher hydrolytic efficiencies or cleavage of additional sites due to partial unfolding of the protein structure, which can make cleavage sites more susceptible to undergo peptide bond hydrolysis catalyzed by the M-POM.[85,86,89]

4. REDOX ACTIVITY

Understanding the redox activity of POMs toward proteins is of crucial importance since different studies have suggested that the redox properties of POMs are responsible for their antibacterial and antitumor activity.[4,16] For example, the observed reduction of $K_6[P_2W_{18}O_{62}]\cdot 14H_2O$ and $K_4[SiMo_{12}O_{40}]\cdot 3H_2O$ in Gram-positive drug-resistant bacteria is an indication of their ability to penetrate through the cell wall and interfere with the electron transfer system of the cell, making these POMs effective antibacterial agents.[92] Furthermore, the ability of POMs to oxidize biological substrates through activating H_2O_2, which is akin to the reactivity of peroxidase enzymes, has enabled their development as biosensors for the detection of H_2O_2 – such as in colorimetric immunoassays for cancer cells—and as catalysts for the degradation of dye pollutants in water.[53,93−101] Therefore, the multiple emerging biological and medical applications of POMs incite studying the redox chemistry of POMs toward biomolecules.[4,16,102]

POMs are powerful oxidation catalysts since they are composed of several early transition metal centers in their highest oxidation state.[103,104] Moreover, these metal centers can be reversibly reduced multiple times, allowing them to engage in many different types of redox reactions involving single or multielectron transfers.[104] This allows POMs to act as catalysts in oxidation reactions with a wide range of different substrates. These redox reactions generally occur through either direct or indirect oxidation modes of action. Direct oxidation involves a redox reaction between the substrate and the POM scaffold or a redox active metal cation coordinated to the POM. On the other hand, indirect oxidation can take place when the POM is used to activate another oxidant (e.g., H_2O_2, O_2, O_3, N_2O, etc.) or to generate reactive oxygen species (ROS) that then oxidize the substrate.[92,105−108] Both of these modes of action have significant potential in the use of POMs for promoting oxidative modifications of proteins. Moreover, the structural versality of POMs allows for the redox properties to be tuned through the type of metal centers,[103,109−111] the counterions,[112−114] and the overall charge,[115,116] as well as the solvent in which the reaction is performed.[117] However, the ability of POMs to engage in redox reactions with proteins has been scarcely explored so far. Therefore, to prompt further research in this direction, key examples of reported studies on the redox activity of POMs toward proteins and the potential mechanism of these reactions are discussed in the following section.

4.1. Direct Oxidation of Amino Acids, Peptides, and Proteins

In nature, proteins participate in several types of redox reactions mainly due to the presence of redox-active amino acids, such as cysteine (Cys), methionine (Met), tyrosine (Tyr), tryptophan (Trp), histidine (His), and phenylalanine (Phe).[118] These amino acids, with the exception of Met, have been recently reported to induce the reduction of Ce^{4+} into Ce^{3+} in Ce-K 1:2 or Ce-WD 1:2 M-POMs (Figure 6).[80,119] Furthermore, the rate of reduction of Ce^{4+} was found to be significantly influenced by the reaction conditions, the POM scaffold, and the nature of the amino acid substrates. In general, increasing the pH, ionic strength or the temperature

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enhanced the rate of reduction of Ce^{4+}. The rate of reduction was also observed to be 1 order of magnitude faster for Ce-K 1:2 compared to Ce-WD 1:2, in line with the more positive redox potential of Ce-K 1:2. In addition, Ce^{4+} was determined to be most susceptible to reduction in the presence of Cys, leading to the formation of cystine via a radical homocoupling mechanism (Figure 6). In contrast, reduction of Ce^{4+} with other redox active amino acids did not afford clearly detectable oxidation products, suggesting that the solvent molecules are involved in regenerating the amino acid. Interestingly, incorporation of these amino acids into di- or tri- peptides decreased the rate of reduction of Ce^{4+}, which was proposed to be due to changes in the redox potential and steric environment of the amino acids. Furthermore, although reaction intermediates were not identified, coordination of Ce^{4+} to the amino and/or carboxylate group were determined to be important for the reaction to take place. Nevertheless, no hydrolytic fragments were detected, indicating that the redox reactions were faster than the hydrolytic cleavage.

The redox activity of M-POMs toward proteins has also been reported and showed a clear complementarity to the hydrolytic activity. Incubation of Ce-K 1:2 with hemoglobin was observed to result in the reduction of Ce^{4+} to Ce^{3+}, with a higher reduction efficiency at pH 7.4 compared to at pH 5.0. Conversely, lower hydrolytic activity was observed at pH 7.4, which was ascribed to the faster reduction of Ce^{4+} into Ce^{3+} at higher pH.[81] This curious interplay between the redox and hydrolytic activities was also evident from the differences in the extent of hydrolysis of the α- and β-chain units of hemoglobin. While the β-chain was cleaved at five different positions, only two cleavage sites were detected for the α-chain. The lower number of cleavage sites observed for the α-chain was attributed to a greater presence of redox active histidine residues in POM-accessible random coils of this unit compared to the β-chain, which caused the reduction of the hydrolytically active Ce^{4+} to the hydrolytically inactive Ce^{3+} around the α-chain.[81] Therefore, although the redox activity of Ce^{4+} M-POMs can hinder their ability to induce protein fragmentation, this opens the door to protein cleavage and modification mediated by oxidation reactions.

4.2. Indirect Oxidation of Proteins

In addition to the reported direct oxidation reactions of amino acids, peptides and proteins by Ce^{4+} M-POMs, a recent report by our group has shown that a Cu^{2+} M-POM ($[Cu^{2+}(H_2O)(\alpha_2\text{-}P_2W_{17}O_{61})]^{8-}$; Cu-WD) can be used for the selective indirect oxidation of proteins (Figure 7). Cu-WD was shown to induce regioselective oxidative cleavage of lysozyme in the presence of ascorbate under physiological conditions (pH 7.5, 37 °C).[120] As indicated by LC MS/MS analysis, SC XRD, and an array of control experiments, the selectivity was proposed to be due to interactions between positive patches of the protein and the negatively charged POM scaffold. These interactions positioned the Cu^{2+} center at specific regions of the protein surface, and, therefore, the production of ROS occurred only in the vicinity of the binding sites (Figure 7). Such controlled oxidative cleavage of proteins can be used to understand the impact of oxidation on protein structure and function, which is of significant interest for biomedical research given that oxidative modifications have been linked to diseases like Alzheimer's and cancer.[121,122] Hence, as can be seen from these seminal results, combining the selectivity of POM-protein interactions with the redox activity of POMs can be leveraged as a powerful tool for selective oxidative modifications of proteins. Therefore, further research into the redox activity of POMs toward proteins could provide valuable insights to address important challenges in medicine.

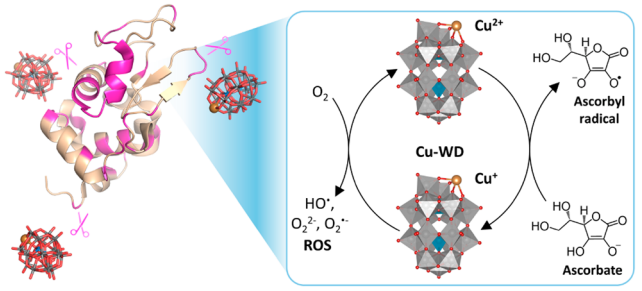

Figure 7. Crystal structure (PDB code 6HY8) of lysozyme with $([Cu^{2+}(H_2O)(\alpha_2\text{-}P_2W_{17}O_{61})]^{8-}$ (Cu-WD) showing the oxidative cleavage sites in magenta (left), which are due to the formation of reactive oxygen species (ROS) induced by Cu-WD and ascorbate in the vicinity of the binding site, resulting in protein fragmentation.

5. OUTLOOK AND OPPORTUNITIES

Significant efforts have been made over the past few years to shed light on the interactions and reactivity of POMs toward proteins. Clearly, elucidating the structure−activity relationships of POMs in these reactions is a complex endeavor due to the interplay between shape, size, charge, and composition of both the POM and the protein along with the influence of the solution conditions, which also needs to be considered. Hence, obtaining valuable insights requires a multitechnique approach combining computational modeling, crystallography, and systematic spectroscopic studies, which is essential for the development of future POM catalysts. Spectroscopic methods can be used to obtain valuable experimental information about the species present in solution, as well as how they interact and react with each other (e.g., POM speciation, protein conformation, binding affinity, reaction rate, etc.), while crystallographic and computational studies can provide molecular-level mechanistic information (e.g., solid-state structure, binding sites, mode of binding, possible reaction pathways, etc.).

Despite remarkable advances such as the ones described in this perspective, important challenges remain in order to enhance the efficacy of these metal-oxo clusters. For example, to enable the implementation of POMs as a new generation of artificial proteases, especially for the analysis of protein fragments in the context of proteomics, the water-soluble POMs typically used as homogeneous catalysts need to be separated from the solution containing the desired peptide fragments. Therefore, new strategies should be developed for the fast and efficient removal of POMs from the formed peptide mixture. Alternatively, the design of M-POMs that could act as heterogeneous catalysts in peptide bond hydrolysis would be an interesting direction of future research.[123] As a result, further developments should ideally bridge the gap between model studies and real-world applications through reaction optimization. In addition, the multitechnique approach employed for the studies with proteins has proven particularly powerful and could ideally be expanded to other biomolecules, such as nucleic acids, nucleotides, and sugars toward which POMs have already been shown to also display promising reactivity.[124−127]

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Moreover, there is still a need for more detailed molecular-level studies to gain a deeper understanding of the reaction mechanisms, particularly with respect to the redox activity of POMs toward proteins. This type of reactivity remains so far understudied despite the redox activity of POMs being extensively investigated in other areas.[128] Therefore, further research into the hydrolytic and redox activity of POMs toward proteins, and biomolecules in general, is needed to further understand the therapeutic activity of POMs and enable their use in bioanalytical methods. Furthermore, there is still significant unexplored potential in the use of covalent functionalization of POMs with different organic groups to tune and direct the reactivity. This is particularly important for the design of POMs as therapeutic agents since their clinical applications are currently limited by their low biocompatibility and selectivity, as well as their relatively high off-target toxicity, which can be remediated through covalent functionalization or incorporation into composite materials.[4] Hence, research in this direction could open new biological and/or medical applications of POMs.

Finally, the reactivity between POMs and proteins discussed herein has already inspired research on other promising reactivity avenues, which have been emerging in recent years. For example, some of these results are being translated into the use of other types of metal-oxo clusters as discrete units, or in MOFs, for the hydrolysis of proteins, and may be extended to other biological systems.[13,129,130] Additionally, metal-oxo clusters in general have been shown to serve as excellent and highly robust catalysts not only for the cleavage of amide bonds, but also for their formation, which is instrumental for the synthesis of certain pharmaceuticals, polymers and other important synthetic compounds.[131−133] Therefore, the study of the reactivity between POMs and proteins addresses under-explored areas of bioinorganic chemistry, and paves the way toward varied innovative solutions across chemistry.

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Author Contributions

‡D.E.S.M., N.D.S., S.A.M.A., and F.d.A. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. CRediT: **David E. Salazar Marcano** conceptualization, writing-original draft, writing-review & editing; **Nada D. Savić** conceptualization, writing-original draft, writing-review & editing; **Shorok Abdelhameed** conceptualization, writing-original draft, writing-review & editing; **Francisco de Azambuja** conceptualization, writing-original draft, writing-review & editing; **Tatjana N. Parac-Vogt** conceptualization, resources, supervision, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank KU Leuven and the Research Foundation Flanders (FWO) for funding. D.E.S.M. (1183021N), N.D.S. (1267623N), F.d.A (1281921N), and S.A.M.A. (1115321N) thank the FWO for fellowships.

ABBREVIATIONS

DFT, density functional theory; HPOM, hybrid polyoxometalate; HSA, human serum albumin; ITC, isothermal titration calorimetry; LC MS/MS, liquid chromatography tandem mass spectrometry; MOF, metal organic framework; M-POM, metal-substituted polyoxometalate; pI, isoelectric point; POM, polyoxometalate; ROS, reactive oxygen species; SC XRD, single crystal X-ray diffraction

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