Introduction and Disclaimer

These mock examination questions span diverse disciplines and are designed for your practice in preparation for the International Research Olympiad (IRO) 2024. Endeavor to answer them to the best of your ability, utilizing this opportunity to enhance your skills and knowledge. For additional practice, it is advisable to engage in extensive reading of various papers; such efforts will contribute to a more comprehensive and nuanced understanding of the subject matter.

Try your best, and good luck! –International Research Olympiad 2024

Artificial enzymes with multiple active sites

Abstract

Artificial enzymes based on the modification of a protein structure for the creation of new active catalytic sites have experienced a great boom in recent years. Multidisciplinary strategies of genetic engineering, chemical or chemical biological tools have been successfully described to synthetize them. However, a challenge has been focused on the creating of artificial enzymes with more than one active site. This could represent a new direction in the application of enzymatic tools in sustainable chemistry. Actually, only a few technologies have been described for designing artificial enzymes with two or multiple active sites. This review article underlines these most significant advances.

Introduction

From the viewpoint of sustainable chemistry, a multi-step transformation enabled by multiple catalysts in one pot is quite attractive. This is because it can improve the overall yield and reduce the use of chemicals such as organic solvents by omitting laborious isolation of the reaction intermediates.

One of the most advantageous systems described in the last years has focused on the development of combining catalysts, which allow producing more selective and efficient compounds, reducing environmental problems. This is the particular case of the cooperative of enzymes or enzymes with a metal complex catalyst. This latter allows the combination of the high reaction selectivity of enzymes with the reaction repertoire of metal complexes. However, this is challenging because these two types of catalysts are often mutually inactivated and the reaction conditions for one cannot be applied to the other.

In this term, artificial enzymes have been representing a novel alternative. Artificial enzymes are based on a modified starting protein structure by creating new active sites in its structure. This is done by combining multidisciplinary strategies of genetic engineering, chemical or chemical biological tools. Thus, to date, different types of artificial enzymes that mimic or enhance enzymatic or unnatural activities have been successfully created.

In this regards, strategies such as genetically modification of amino acids altering the function or modifying the metal-coordinated amino acids environment in natural metalloenzymes, incorporation of metals complexes in supramolecular systems with strong affinity, creating new enzymes activities or nonbiological ones from noncatalytic protein scaffold or the replacement of the existing metal ion with an alternative one has been developed.

However, one of the most important questions in green chemistry is the combining of both catalysts in aqueous or cosolvent media. In most cases, this is a limitation of the system for developing the cascade process, which needs to combine the aqueous with the nonaqueous media.

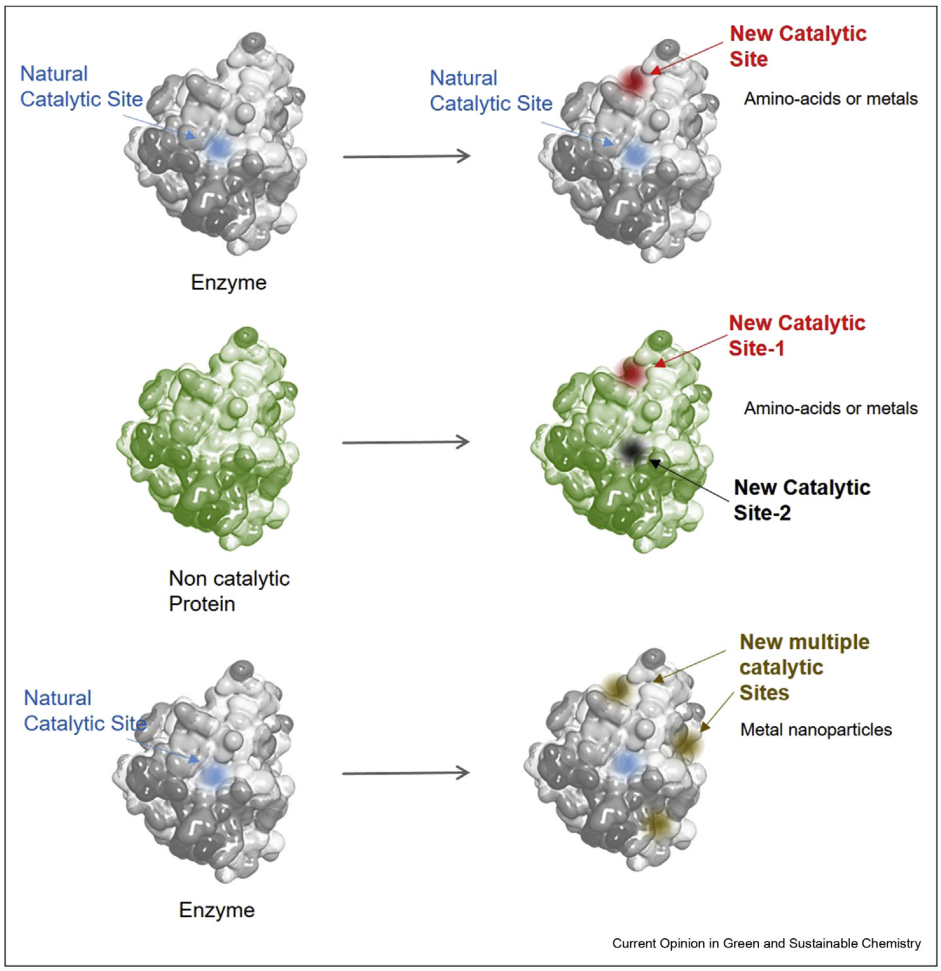

Figure 1: General concept in the creation of novel enzyme with multi activesites.

For this reason, the most challenging would be to have both active catalytic sites in the same compartment. Nevertheless, important issues must be considered, such as the selection of the desired protein scaffolds, position of the new sites, compatibility with the existing active site, relative distances between multiple sites in order to perform the chemical processes adequately.

In particular, artificial enzymes with more than one active site represent a challenge in modern sustainable chemistry. Also, the possibility to obtain more sustainable artificial enzymes goes through reuse as a heterogeneous form.

In this review, the most recent strategies about the creating of artificial enzymes with more than one active site, or heterogeneous multiactive sites artificial enzymes are discussed (Figure 1).

Artificial metalloenzymes by selective creation of a novel active site in an enzyme scaffold

One of the strategies to create artificial enzymes considers the existence of a natural enzymatic three-dimensional cavity in the protein scaffold. The insertion of organometallic complexes directly on the active site of enzymes or alteration of natural metallic complexes on enzymes by genetic tools has been successfully used for creating new enzymes with excellent new synthetic applications. This presents the disadvantage that enzymatic activity is completely killed. However, the most fundamental awareness will be the possibility to create enzymes (proteins with intrinsic activity) with other additional catalytic activity.

In this regard, in my group, we designed for the first time an enzyme with two active sites, using a natural characteristic of the thermophilic *Geobacillus thermocatenulatus lipase*, one of a few lipases with two lids. The spatial area between these two oligopeptide chains (lids) was discovered as a natural new active site. Introducing a unique cysteine in one of the lids by genetic modification, for site-specific Cu coordination ligand, we created a new abiological place. Both active sites were effective in hydrolysis and cycloaddition activity and worked properly by cascade combining hydrolase + reductase. Another advantage of this two-catalytic system is that this was selectively performed on solid-phase, introducing advantages of recyclability.

Based on this idea, recently Ferrer and coworkers designed an elegant strategy by genetic and computational way of a hydrolase with two different active sites. Different optimization of the amino acid groups and the position was necessary to selectively incorporate an irreversible inhibitor mimics Cu molecule in the natural active site, leaving free the newly created catalytic triad (Ser211, His214, Asp25) (Figure 2b). This advantage allowed the creation of an artificial enzyme containing an enzymatic hydrolytic site and a chemo Cu-site. This new enzyme with two active sites was successfully applied in cascade reactions, such as the enzymatic hydrolysis of vinyl acrylate followed by a Friedel-Craft alkylation reaction of the produced acid, to finally yielding enantiomerically pure (S)-3- phenylbutyric acid (Figure 2b). One of the most important processes in many cascade reactions is based on the critical problems with the need for using cofactors and the regeneration of them. In this term, artificial enzymes incorporating the capacity for performing the enzymatic reaction of interest but also recovering the cofactor will be a challenge.

At this point, recently Duan and coworkers designed a novel artificial enzyme-based host-guest supramolecular strategy to combine abiotic and biotic catalysts *via* NADH-containing host, eliminating the inherent communication barrier and interference between both systems. This strategy offers the key advantages of supramolecular catalysis in integrated chemical and biological synthetic sequences. The strategy consisted in the incorporation of a Zn-ZPA complex, previously obtained from the reaction between $Zn(BF_4)_2 \cdot H_2O$ and H_2ZPA, a ligand containing a dihydropyridine amido mimic similar to the active site of an NADH model with a pendant C(O)–NH group as potential hydrogen-bonding sites, in the catalytic site pocket of formate dehydrogenase (FDH) enzyme (Figure 2c). In this case, a flavin-dependent monooxygenase-like activity from the organometallic complex acted synergistically with enzyme hydrogenase activity in the BaeyereVilliger reaction avoiding the necessity to cofactor regeneration, being the process *in situ*.

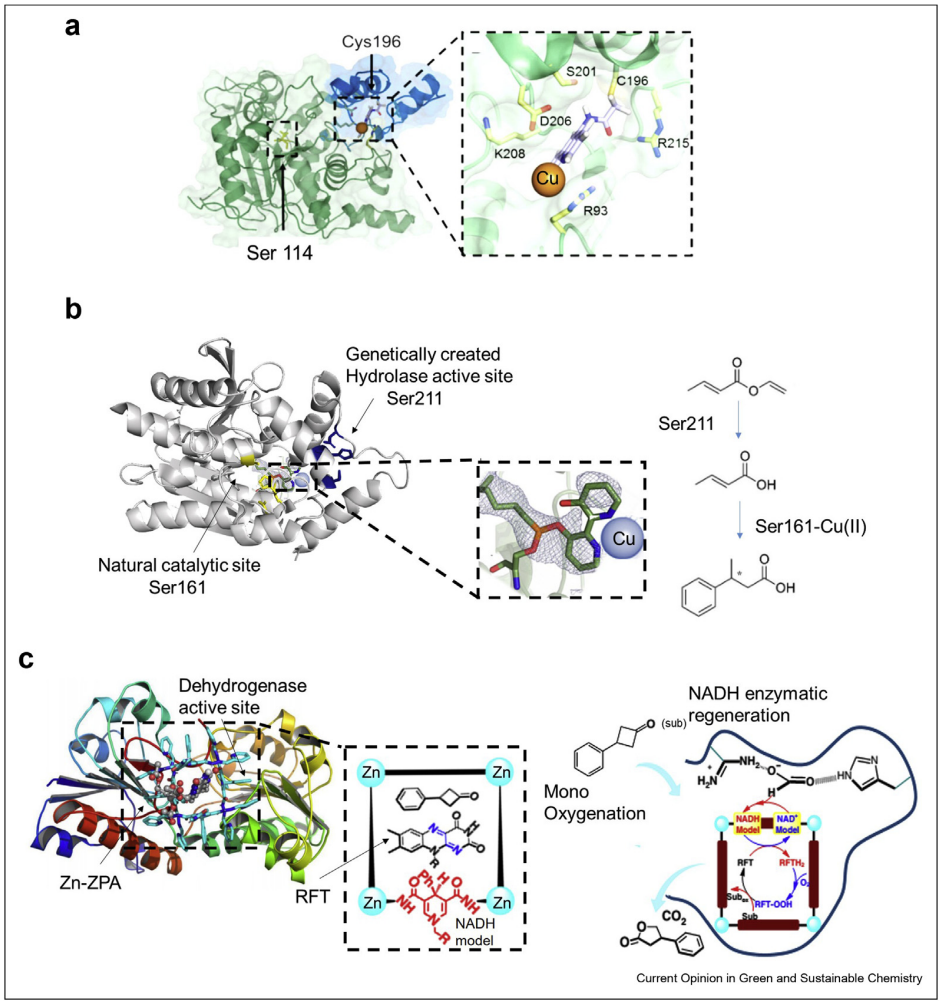

Figure 2: Designed and application of artificial enzymes with two active sites (abiotic and biotic). (a) Creation of a natural cavity in G. thermocatenulatus lipase (GTL) by genetic and chemical Cu binding ligand. (b) Genetically created a new active site in a hydrolase for two catalytic binding pockets applied in cascade reaction. Natural catalytic serine 161 was modified by Cu binding by irreversible inhibitors strategy. (c) Creation of a cofactor active site in the cavity pocket of activated site of formate dehydrogenase (FDH) for selective Baeyer–Villiger reaction. The protein structures were obtained from the Protein Data Bank (PDB code: 2w22 (A), 6I8F (B), and 2FSS (C), and the pictures were created using Pymol vs 0.99.RFT: riboflavin tetraacetate.

Artificial enzymes by creating abiological active sites using a noncatalytic protein as a scaffold

The use of a protein scaffold (no biological activity) for the creation of artificial enzymes has been one of the precious, elegant methods. Using covalent, supramolecular interactions such as protein-molecules and other interesting scaffolds have been described.

Particularly, in the creation of copper artificial metalloenzymes, a very fancy example was developed a few years ago using the Cu-binding ligand in a particular protein linear beta-sheet structure for stereoselective chemistry. Very recently, a new approach by using supramolecular coordination of Cu complexes into the cavity of the transcription factor Lactococcal multidrug resistance Regulator (LmrR), a homodimeric protein an unusually large hydrophobic pore at the dimer interface, with a single mutation produced an interesting metalloenzyme catalyzing the tandem Friedele-Crafts alkylation/enantioselective protonation of indoles with a-substituted enones with $>$80 yields and 59% ee (Figure 3a).

Although this represents an excellent example of how to create synthetic enzymes, so far, one more step has been determined by the same group, where Roelfes and co-workers have designed the first artificial enzyme containing two abiotic sites in a noncatalytic protein scaffold.

A complex strategy has been developed where a catalytically active Cu(ii) complex and organocatalytic unnatural amino acid were successfully introduced in a dimeric LmrR (a noncatalytic protein scaffold) (Figure 3b). For that, there were two important matters of the design. One was the promiscuity of the hydrophobic cavity of LmrR, where the binding of the Cu(ii)-bound enolate between central tryptophan residues W96/W960 is of key importance both for the activity and selectivity of the reaction. The second was the physical separation and careful locating of the cu(ii) complex and p-amino-phenylalanine (pAF) residue (genetically introduced at position 15), which avoid problems related to the incompatibility of the individual catalytic components, while allowing for an efficient and selective approach of the nucleophile to the activated electrophile.

This new artificial enzyme with two different abiotic sites was successfully tested in Michael addition reaction where the pAF activates an enal through iminium ion formation whereas the Cu site (Cu(ii) complex) activates the Michael donor by enolization and delivers it to one preferred prochiral face of the activated enal (Figure 3b). These two sites act synergistically to achieve high activity and enantioselectivity. An optimization of the protein by introducing a new mutation in 8 positions making light a more hydrophobic environment by changing Met residue by Leu residue improved the efficiency of both abiotic catalytic centers, improving yield and enantiomeric excess (Figure 3b).

Thus, this technology could be extended to other metals and organocatalytic unnatural amino acids, providing an attractive way to advance in the application of enzymatic catalysis in chemistry.

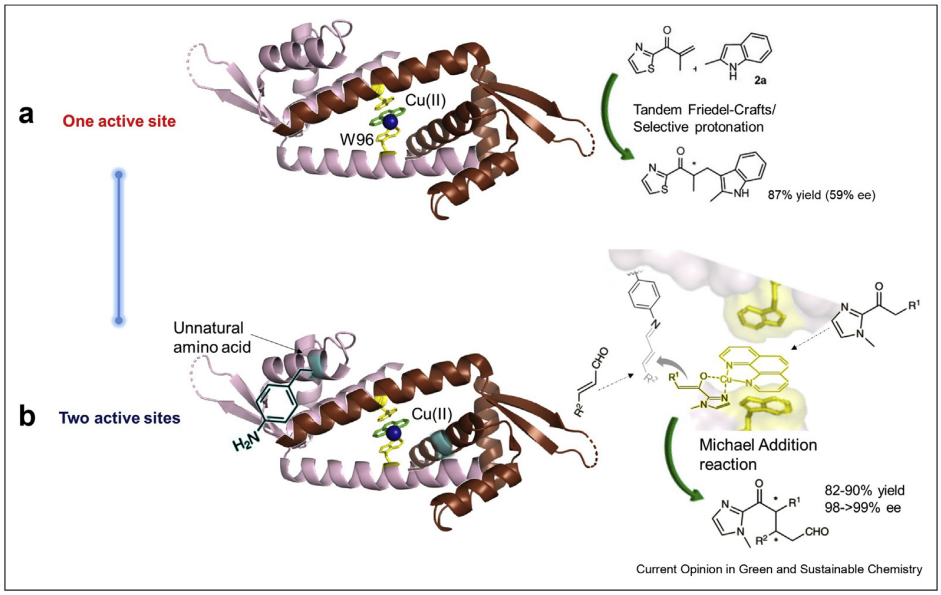

Figure 3: Artificial enzymes with abiological active sites. (a) Artificial metalloenzyme Cu-LmrR with one active site applied in C–C bonding reaction. (b) Artificial metalloenzyme containing two active sites (non-natural amino acid + Cu sites) for selective Michael Addition.

Artificial enzymes by multiple active sites by enzyme-metal nanoparticles hybrids

The insertion of an organometallic structure on a protein cavity has been demonstrated in the previous examples as an excellent strategy to create additional active sites in a proteic system. However, the complexity of the organometallic compounds, the need to use protein engineering and design, or the successful production of protein in large amounts seem to be some important limitations for a possible industrial application of these artificial enzymes. Between all these drawbacks, the matters related to the effectiveness, capacity, and selectivity in the introduction of each new active site and their final synergistic effect must also be taken into account. Therefore, considering all these shortcomings, recently, we have developed a challenging strategy that allowed us to produce a high amount of artificial enzymes containing multiple active sites in the same protein matrix. The novel strategy is based on the creation of homogeneously dispersed very small metal nanoparticles in situ in a protein matrix of an enzyme, where the enzyme has a key role on that, acting as a reducing agent (producing the nanoparticles nucleation) but also as a stabilizer of these metal nano-particles (Figure 4). The advantage of this system is that we can obtain multiple metallic, active sites in an enzymatic network where the native enzymatic activity is still conserved. Therefore, we obtain, by a very efficient and mild strategy, a high amount of a heterogeneous artificial metalloenzyme material with multiple active sites (metallic and enzymatic) in the same compartment. The methodology is extremely versatile, allowing the combination of different metals or different enzymes for obtaining multiple active sites. As multiple, metallic active sites, these enzyme-metal nanoparticles hybrids have been used excellently in 6 Synthetic Enzymes many different chemical applications, improving chemical processes (e.g CeC bonding reaction) also in more sustainable conditions, for example, in aqueous media or water-cosolvent systems, or moderate T. However,

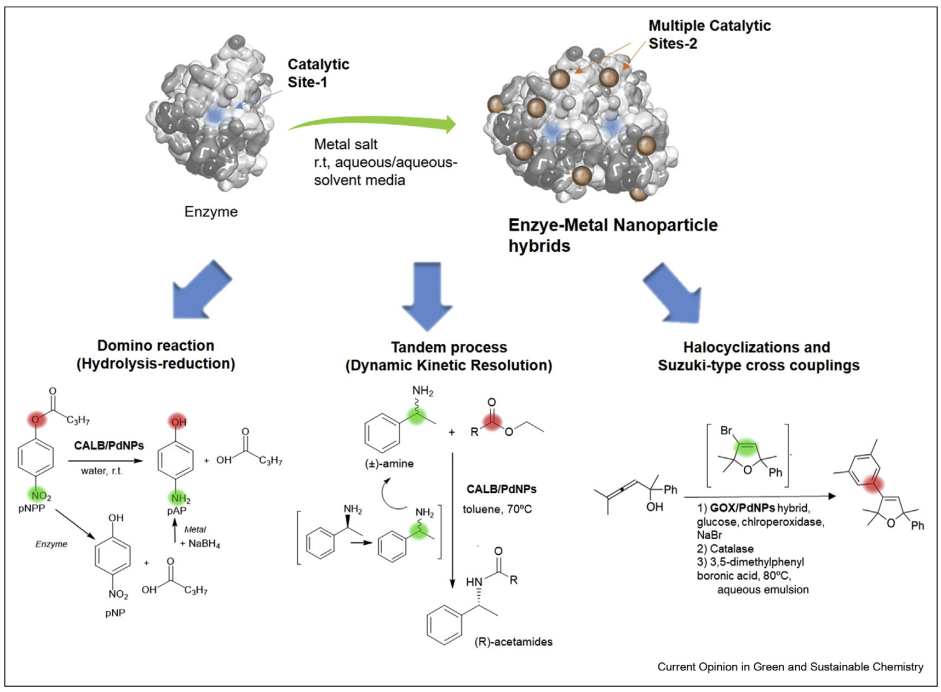

Figure 4: Design and application of novel metalloenzymes multiple active sites. Reaction performed by enzymatic catalytic site (green). Reaction performed by metallic catalytic site (red).

the real advance of these novel artificial metalloenzymes hasve been demonstrated in efficient cascade processes by synergistic dual activities, natural enzymatic activity (catalytic site 1), and the multiple metallic nanoparticles (catalytic sites 2) (Figure 4). The combination of hydrolytic activity of the enzyme CALB and the reducing activity of Pd nanoparticles made possible the one-pot transformation of p-nitrophenyl propionate in p-aminophenol in water and room temperature by the heterogeneous biohybrid (Figure 4). Using the same enzyme with Pd nanoparticles, an asymmetric application ddynamic kinetic resolution of racemic amined was demonstrated. The CALB/PdNPs hybrid was successfully used for a one-pot tandem catalysis to produce enantiopure (R)-benzylamide (ee>99%, c:98%), combining the high enantioselectivity of the enzyme and the high racemization capacity of PdNPs (Figure 4). These results also showed the versatility of these multiple catalytic materials because the reaction was performed in toluene at 70 °C. Additionally, these heterogenous catalysts showed excellent stability being recyclable several times in the different processes without loss of catalytic efficiency. Very recently, this potential ability in cascade multiple catalysts has been validated in arylative allenol cyclization. A novel artificial enzyme constituted by glucose oxidase (GOx) and Pd nanoparticles was synthesized and applied in a one-pot reaction in aqueous media at room temperature. In the first step, the bio-catalytic halocyclization of allenic alcohol was obtained by a chloroperoxidase via the consumption of H2O2 in situ produced by GOx, which, in a second step, was transformed by a Suzuki reaction catalyzed by the PdNPs in the desired product in excellent enantiopurity (Figure 4). These examples demonstrated the excellent applicability and the future of this methodology in sustainable organic chemistry. Indeed, combining the high capacity of the chemical complexity of some lipases with excellent properties and the broad range of metal catalytic reactions could be an excellent strategy to address important complex synthetic problems.

Concluding remarks

Artificial enzymes containing two or more active sites in the same protein scaffold represents a challenge in the biocatalysis and biotechnology industry. Actually, there are only a few examples of successful results in creating these such biocatalysts, which have been discussed in this review. However, although these systems are still in infancy, the present work demonstrates the extremely high potential application of these novel artificial enzymes in the development of more environmentally friendly and sustainable chemical processes. Indeed, for the first time, the methodology of the enzyme-metal hybrids ensure the possibility to achieve multiple active site catalysts at large-scale and in heterogeneous form, for a realistic potential industrial implementation and with an important social-economic effect.

Paper 4: Biochemistry

Question 1

What are key advantages of artificial enzymes with multiple active sites?
(Select all that apply)

a) They allow for reactions to occur at extreme temperatures

b) They allow for cascade reactions in one pot without isolating intermediates

c) They allow for the incorporation of toxic metals safely

d) They have stability which allows for multiple uses

Question 2

How was the first artificial enzyme created with both a natural and an abiological catalytic site?
(Select one)

a) Computationally designing a new protein structure

b) Mutating residues randomly until a site is formed

c) Genetically modifying a lipase to introduce a new Cu binding site

d) Incorporating a metal complex into a cavity in the structure

Question 3

Which of the strategies would mostly likely be difficult for researchers when trying to design an artificial enzyme with multiple catalytic sites?
(Select one)

a) Ensuring proper spacing between catalytic sites

b) Introducing cofactors into the cavities

c) Stabilizing the enzymes to prevent degradation

d) Achieving high yields

Question 4

Which of the following factors would be most important to consider for a protein scaffold when designing an artificial enzyme for pharmaceutical applications?
(Select all that apply)

a) Thermal stability

b) Shelf life

c) Immunogenic potentials

d) Solvent tolerance

Question 5

Which types of chemical reactions would be most suitable to perform using an artificial enzyme?
(Select all that apply)

a) PCR amplification

b) Gabriel synthesis

c) Pericyclic reaction

d) Michael addition

Question 6

Which spectroscopic technique is *least* useful in providing insight about an enzyme-metal nanoparticle hybrid?
(Select one)

a) UV-Vis (Ultraviolet-visible spectroscopy)

b) CD (Circular dichroism) spectroscopy

c) EXAFS spectroscopy (Extended X-ray absorption fine structure)

d) Raman spectroscopy

Free Response Question 1

What aspect of enzyme-metal nanoparticle hybrids make them suited for sustainable chemistry?

Free Response Question 2

What are two different strategies discussed in the paper for creating artificial enzymes that have multiple catalytic sites? For each strategy, explain how these sites are formed, and provide an example from the paper.