Probing the Electrocatalytic ORR-Reactivity of Immobilized Multi-Copper Oxidase CueO

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ABSTRACT The bioelectrocatalytic (oxygen reduction reaction, ORR) properties of the multi-copper oxidase CueO immobilized on gold electrodes were investigated. Macroscopic electrochemical techniques were combined with *in-situ* scanning tunneling microscopy (STM) and surface enhanced Raman spectroscopy (SERS) at the ensemble and at the single molecule level. Self-assembled monolayer of mercaptopropionic acid, cysteamine and p-aminothiophenol were chosen as redox mediators. The highest ORR activity was observed for the protein attached to amino terminated adlayers. In-situ STM experiments revealed that the presence of oxygen causes distinct structure and electronic changes in the metallic centers of the enzyme, which determine the rate of intramolecular electron transfer and, consequently, affect the rate of electron tunneling through the protein. Complementary Raman spectroscopy experiments provided access for monitoring structural changes in the redox state of the T1 copper center of the immobilized enzyme during the CueO-catalyzed oxygen reduction cycle. These results

unequivocally demonstrate the existence of a direct electronic communication between the electrode substrate and the Type 1 copper center.

KEYWORDS: Laccase, cysteamine, p-aminothiophenol, protein film voltammetry, oxygen reduction, gold electrode

INTRODUCTION

The immobilization of enzymes on electrode surfaces to catalyze complex electrochemical reactions was originally proposed by Tarasevich et al.^{1,2} Since then, much research has focused on the elucidation of methods for the immobilization of enzymes to optimize their electrocatalytic activity.³⁻⁸ Key factors are (i) the interaction of the enzyme with the electrode surface, which may lead to denaturation, (ii) the orientation on the surface as well as (iii) the positioning of the active site of the enzyme such that it remains accessible for solution species.⁹ Moreover, electroactive enzymes contain a redox active moiety that should remain close enough to the electrode surface to allow fast and effective redox communication. Such communication can be achieved through direct electron transfer or by the indirect action of redox mediators. The direct electron transfer pathway is always desirable since the use of mediators leads typically to thermodynamic losses.¹⁰

The best catalysts for oxygen reduction and hydrogen oxidation known to date are based on platinum group metals.¹¹ However, the scarcity and the high prize of platinum are major obstacles for a broad commercialization, for instance in fuel cell technology.¹² Attempts to search for novel catalysts based on more abundant first row transition metals lead to

hydrogenases (Fe^{2+} and Ni^{2+} redox centers) and oxidases ($\text{Cu}^{2+/+}$ redox centers) as attractive model systems.¹³

Multi-copper oxidases, such as laccases, ascorbate oxidase, ceruloplasmin, bilirubin oxidase and CueO (copper efflux oxidase), represent a family of enzymes that catalyze the reduction of oxygen.^{14,15} Despite their different structures, they are all characterized by possessing two active centers formed by 4 copper atoms. The copper sites are classified according to their spectroscopic properties.^{15,16} Type 1 or blue copper sites are the active sites for substrate oxidation. In them, the copper atom is coordinated to one cysteine (Cys) and two histidines (His), whereas the fourth ligand varies, depending on the enzyme. Most often it is an axial methionine (Met). The blue copper site shows a strong UV/Vis absorption band at 610 nm. The second active center is composed of three copper atoms, one called Type 2 and the other two are classified as Type 3. The Type 2 copper atom is coordinated to two histidines (His) and one water or OH-species. It is not UV/Vis active, but EPR (electron paramagnetic resonance) active. The two Type 3 Cu atoms are characterized by their coordination to three histidines (His) and bridging OH-species and they exhibit a characteristic UV/Vis band at 330 nm. The trinuclear Type 2/3 center is the active site for oxygen reduction. Electrons are transferred from the Type 1 copper to the Type 2/3 copper center through a chain of His-Cys-His amino acids, where they react with oxygen leading to a complete reduction to water. Protons are provided from acidic amino acids (aspartic acid, Asp112 in the case of CueO), which are located close to the Type 2/3 copper center.^{17,18}

Multi-copper oxidases have been immobilized on electrodes by direct adsorption or via mediators.^{3,19-25} For the bioelectrocatalysis, the natural substrate is substituted by the electrode as the source of electrons. The entry point of electrons is typically attributed to the Type 1

copper²⁶. However, recent spectro-electrochemical experiments provide evidence that electrons may also enter directly into the Type 2/3 center.²⁷ Ramirez et al. suggested that the entry point depends on the electrode material, most likely because the latter may affect the surface orientation of the enzyme.⁹

The multi-copper oxidase CueO as found in *Escherichia coli* catalyzes the conversion of Cu(I) into the less toxic Cu(II).²⁸⁻³⁰ Figure 1 shows the tridimensional structure of CueO protein together with a detailed view of the active center.^{29,30} The presence of a methionine-rich alpha helix, that covers the region close to the Type I copper center, leads to a low laccase activity.²⁸ Depletion of the methionine rich chain increases the laccase activity but decreases the cuprous oxidase activity.³¹ This alpha helix is close to the binding site of a fifth copper atom, which seems to be of key importance for the activity of the oxidase. The fifth (regulatory) Cu atom is ligated to two methionines, two aspartic acid residues and one water molecule.³⁰

Figure 1: Tridimensional structure of CueO (pdb structure 1N68). (A) Surface view showing the distribution of acidic (red: Asp, Glu), basic (blue: Arg, Lys, His), hydrophobic (grey: Ile, Leu, Phe, Val) and sulphur containing (yellow: Cys, Met) residues.(B) cartoon view illustrating the methionine-rich alpha helix close to the Cu Type 1 center. (C) Stick view showing the amino acids around the active center.

CueO has been successfully immobilized on carbon electrodes showing a distinct oxygen reduction activity.³²⁻³⁵ The present paper explores the role of three self-assembled monolayers as mediators for the ORR of CueO. The mechanism of the enzyme-catalyzed ORR is explored employing *in-situ* scanning tunneling microscopy/spectroscopy (STM, STS) and surface-enhanced Raman spectroscopy. New information about structural changes taking place on the

immobilized protein upon electron transfer is revealed with the STM study. On the other hand, the spectroscopic investigation has provided novel information about the orientation of the protein and the route of electron transfer through the protein from the electrode surface to the oxygen substrate.

RESULTS

Voltammetric experiments

Figure 2 displays characteristic cyclic voltammograms of CueO immobilized on bare and SAM-modified Au(111) surfaces in the absence and in the presence of oxygen. Figure 2A shows the current response of the ORR on Au(111) with and without CueO. Comparison of both curves revealed an anodic shift of the ORR onset by about 0.20 V as induced by the presence of the immobilized enzyme. The data demonstrate that the enzyme retains its catalytic activity for oxygen reduction and that the electronic communication between enzyme and the electrode surface is well established without the need of an electron transfer mediator. STM experiments in the presence of oxygen (insert to Figure 2A, for further discussion see below) revealed that CueO is uniformly distributed on the electrode surface without occupying preferential sites. Assigning each bright spot to a surface-confined CueO molecule, we estimate a coverage of $0.50 \text{ pmol cm}^{-2}$ (10 to 15 % of a monolayer) under the chosen assembly conditions. We note that the immobilized CueO molecules are weakly adsorbed and easily pushed away by the STM-tip if the tunneling setpoint current i_T is chosen to be too high ($i_T > 20 \text{ pA}$). The maximum current response for the ORR is achieved after a first conditioning cycle during the second voltammetric cycle (shown in panel A). Further cycling the electrode potential in $-0.15 \text{ V} < E < 0.60 \text{ V}$ leads to a continuous decrease of the catalytic activity, for instance up to 40 % after 1 hour (see Figure S3

in SI for further details). Complementary STM experiments revealed that this decrease in activity is mostly related to the potential-induced loss of immobilized CueO.

In an attempt to improve affinity and stability of the immobilized CueO on Au(111) we investigated next the role of self-assembled monolayers as redox-mediators for the ORR of CueO. Preliminary experiments with hexanethiol (HT), mercaptoundecanoic acid (MUA), mercaptopropionic acid (MPA), cysteamine (CYS), L-cysteine, cystamine, p-aminothiophenol (pATP) and p-mercapto benzoic acid (pMBA) revealed that MPA, CYS and pATP, which are COOH- or NH₂-terminated upon assembly, are the most promising candidates. Alkanethiols with CH₃-solution-exposed end groups as well as pMBA are less efficient. Further, the electrocatalytic activity decreases with increasing chain length. Almost no catalytic activity was observed with ω -NH₂-C₈H₁₆-SH and MUA. MPA, CYS and pATP lead to adlayers, which are either negatively charged (MPA, pK_a(surface)= 5.2-5.6³⁶), positively charged (CYS, pK_a(surface)= 8.65³⁷) or neutral (pATP, pK_a(surface)= 4.9-5.3³⁸), thus allowing to explore three different immobilization concepts.

Figure 2: (A) Cyclic voltammograms (second cycle, 10 mVs^{-1}) recorded with an Au(111) electrode in 0.1 M phosphate buffer (pH=6.5) on the bare gold electrode in the absence (black dotted line) and in the presence (black solid line) of oxygen. The blue dotted line and the blue solid line represent the current response of the Au(111)-CueO electrode in the absence and in the presence of oxygen. The insert shows an STM image of CueO physisorbed on Au(111) with bias = 300 mV and tunneling current setpoint = 60 pA. (B) Time evolution of the catalytic response for CueO immobilized on Au(111)-CYS. (C) Stationary response attained after ca. 10 cycles for Au(111)-CueO in the presence of three different SAMs as redox mediators. The insert of panel C

displays an enlargement of the catalytic response of Au(111)-MPA in the absence (black line) and in the presence (blue line) of CueO.

Following the above immobilization protocols, we observed that the current response of the CueO-catalyzed ORR on the SAM-modified surfaces increases continuously during the initial potential cycles between $-0.15\text{ V} < E < 0.60\text{ V}$ until a steady state is reached, typically after ten sequential cycles with 10 mV s^{-1} . Figure 2B illustrates the evolution of the current response for CueO on Au(111)-CYS. This increase, also observed with the bare gold and the other studied SAM modified surfaces, suggest a reorientation of the protein towards a more favorable position, as induced by polarization at low (more negative) potentials. The potential plays an important role in this activation process since an equivalent waiting time at open circuit potential, or at potentials higher than the onset of oxygen reduction, does not have the same effect. It is unclear at this stage whether this activation is responding to an electrostatically induced reorientation or to a chemical modification of residues in the protein.

Figure 2C compares the steady-state voltammograms of maximum ORR catalytic activity as obtained after ten sequential potential cycles for MPA-, CYS- and pATP-modified electrodes. The response changes in the entire potential range according to the following sequence: $\text{MPA} \ll \text{CYS} < \text{pATP}$. CueO immobilized on pATP-Au(111) is nearly twice as active as compared to CYS as mediator.

Additional experiments with a rotating ring disk electrode demonstrate that the reduction of O_2 is complete to water without H_2O_2 formation (figure S8). Furthermore, the possible participation of spurious Cu^{2+} on the catalysis of the ORR was also tested, demonstrating the absence of activity in solutions with different Cu^{2+} concentration when the protein is not present (Figure S9)

To rationalize the effect of the terminal group of the surface modifier on the catalytic performance of the enzyme, we investigated the chemical nature of the residues on the surface of the protein, classifying them as acidic (aspartic and glutamic acid), basic (arginine, lysine or histidine) and hydrophobic (isoleucine, leucine, phenylalanine or valine). The results are summarized in Figure 1. In addition, Figure 1 marks the sulfur-containing residues as yellow patches. The surface of the protein is composed of a variety of acidic, basic, hydrophobic and sulphur-containing patches. No clear predominance of either kind of residue is observed.

The high stability of immobilized CueO on NH₂-terminated SAMs suggests a preferential binding to acidic surface patches of the protein (red colored sites in Figure 1). In this context we emphasize one particularity in the structure of CueO, which is not found in other laccases. This structure feature is the existence of a binding site for a fifth copper atom close to the Type 1 copper site and buried below a methionine-rich alpha helix.³⁰ The role of this fifth copper atom for the activity of the protein is unclear. However, the laccase activity increases in the presence of an excess of copper suggesting that this copper atom has a regulatory effect. The fourth copper atom is located close to the Type 1 copper center and is ligated by an Asp(360) (Figure 1). Based on the above experimental observations, we suggest that this specific site is likely the region for the attachment of the substrate and the entry point for the electrons to the Type 1 copper. The presence of an acidic residue in this region is most probably the reason for the preference of the enzyme for NH₂-terminated SAMs. SAMs terminated with carboxylate groups seem to lead to a different surface orientation of CueO with the Type 1 copper center located farther from the surface, which results in a less efficient electron transfer.

Another interesting aspect regarding the surface landscape of the enzyme is that the active center is buried under a methionine-rich alpha helix. This sulphur-rich region of the protein

(yellow patches in Figure 1) might explain the ability of the protein to adsorb in a proper orientation on the bare gold surface through the formation of sulfur-gold bonds, leaving the Type 1 center close to the surface.

The stronger binding of CueO to NH₂-terminated SAMs (CYS, pATP) as compared to the COOH-terminated SAM (MPA) as electron transfer mediators is also reflected in the following sequence of electrocatalytic activity for the ORR: Au(111)-MPA < Au(111) < Au(111)-CYS < Au-pATP. In-situ STM experiments (see figure 4 below) revealed that the surface coverage of immobilized CueO follows a similar sequence).

STM measurements

In an attempt to understand the distinct catalytic response of CueO on the three SAM-modified Au(111) electrodes, we carried out a comparative series of complementary *in-situ* STM and Raman spectroscopic investigations. Figure 3A shows the unreconstructed Au(111)-(1x1) at 0.00 V in phosphate buffer, pH ~ 6.5, as obtained after gentle polarization at positive charge densities and electrochemical annealing in chloride-containing electrolyte (for details see ref. 39). The electrode surface is characterized by large, defect-free terraces, which are separated by monoatomic steps. Distinctly different adlayers were obtained upon modification with MPA, CYS and pATP. We note that the uniformity of the respective adlayers increases considerably upon assembly in an argon atmosphere under anaerobic conditions.

Figure 3B shows a typical MPA-modified Au(111)-(1x1) surface in phosphate buffer, pH ~ 6.5. The densely packed MPA-monolayer is characterized by highly ordered molecular domains coexisting with monatomically deep vacancy islands etch pits. Their lateral size varies between 4 to 6 nm effective diameter. The latter are typical for the assembly of alkanethiols and aromatic

thiols on Au(111) surfaces.⁴⁰ The ordered domains are characterized by a regular strip-like pattern, which are composed of three molecular rows aligned in one strip. The ordered adlayer can be represented by a rectangular unit cell with the dimensions $a = (2.3 \pm 0.2)$ nm, $b = (0.50 \pm 0.15)$ nm and $\alpha = (90 \pm 5)^\circ$, as indicated in the inset of Figure 3B. These dimensions lead to a $(8 \times \sqrt{3})$ unit cell. A somewhat similar unit cell was proposed earlier by Petri et al.⁴¹ Assigning each bright spot to a single molecule, one obtains a MPA coverage of 4.3×10^{-10} mol cm⁻². The surface pK_a of 5.2 to 5.6³⁶ suggests that the MPA-SAM is negatively charged towards the electrolyte site. Part of this negative charge could be compensated in the potential region of the ORR (negative charge densities of the Au(111) electrode by solvated cations residing in the electrochemical double layer since the potential of zero charge is slightly more negative than 0.36 V.⁴²)

CYS adsorption increases the mobility of gold surface atoms, which is reflected in distinct structure changes of the substrate. Steps appear more frazzled, and monatomically deep vacancy islands (6 to 12 nm in diameter) occur. Disordered and ordered adlayer regions of 30 to 40 nm effective diameter coexists. The latter are separated by domain boundaries, which follow the main crystallographic directions of the underlying hexagonal substrate (Figure 3C). These adlayers are stable in the entire potential region $-0.15 \text{ V} < E \leq 0.60 \text{ V}$. However, the complex pattern demonstrates that room temperature assembly of CYS does not lead to a uniform, completely equilibrated monolayer under our experimental conditions. The inset to Figure 3C shows details of the ordered CYS layer, which is characterized by a stripe-like pattern composed of aligned bright spots of alternating contrast in adjacent neighboring rows. We identify two characteristic dimensions, the distance of bright spots within each row ($a = 2.1 \pm 0.1$ nm) and the perpendicular distance between every other row ($b = 0.50 \pm 0.1$ nm). Assigning each spot to a

single CYS molecules leads then to a $(7 \times \sqrt{3})$ unit cell composed of 3 molecules. The corresponding CYS-coverage is obtained as $5.0 \cdot 10^{-10} \text{ mol cm}^{-2}$, which is slightly higher than the MPA coverage reported above. We note that the ordered adlayer found in our experiments is similar to results published by Zhang et al.⁴³

Figure 3: In-situ STM-images of the bare and SAM-modified Au(111) electrodes in phosphate buffer, pH ~ 6.5 . The insets display atomic (molecular) resolution images (A) Au(111)-(1x1), (B): Au(111)-MPA. The inset displays also the unit cell of the molecular adlayer. (C): Au(111)-CYS with higher resolved adlayer and unit cell.(D) Au(111)-pATP with high resolution as inset. The typical imaging conditions are: bias = 300 mV, tunneling current = 100 pA for (A), and bias = 250 mV, tunneling current = 150 pA for (B-D).

The exposure of Au(111)-(1x1) to a pATP solution leads to a distinctly different adlayer pattern as compared to MPA and CYS . Figure 3D illustrates the pATP adlayer after a short

assembly time of 15 min under argon in an anaerobic atmosphere. The main image shows an islands-free substrate covered with a disordered adlayer of p-ATP. No etch pit were observed. Long assembly times (> 0.5 h) generate a large number of islands of 0.2 to 0.3 nm in height. Their density increases with time and channel-like structures form after several hours of solution exposure. (see Figure S4 and references^{44,45}). The formation of this percolated network shows no experimental evidence for a 3D etching or deposition. This evolution suggests that pATP is an active component in this restructuring process, eventually giving rise to the local formation of multilayers. The overall pATP adlayer structure is disordered and channel-like (Figure 3D inset).

Next, we investigated CueO immobilized on three Au(111)|SAM- surfaces. Au(111) electrodes modified with MPA, CYS, pATP and subsequently with CueO gave a distinct catalytic response in the presence of oxygen, also in the STM-cell (Figure 4A), which is completely quenched in control experiments under anaerobic conditions in argon (see Figure S5). Single CueO molecules immobilized on Au(111)-MPA appear in an oxygen-saturated electrolyte as bright spots of 5 to 6 nm in diameter with an apparent corrugation height of 0.43 nm (Figure 4B). Such an STM contrast pattern is rather typical for small proteins like CueO.²⁵ We also notice that CueO adsorption on Au(111)-MPA is rather fragile, and even less stable as compared to the bare Au(111) surface (insert in Figure 2A). No preferential adsorption sites, neither at step edges nor at the periphery of etch pits could be identified. Averaging more than ten independent experiments demonstrated that the assembly conditions chosen in this paper led to CueO coverages of $\sim 5\%$ ($0.23 \text{ pmol cm}^{-2}$).

The quantitative analysis of the CueO adlayer structure on Au(111)-pATP is more complex. Short assembly times of 10 to 15 min give rise to a disordered pATP monolayer (Figure 3D), which allows the immobilization of CueO up to a coverage of 25 to 30%. Figure 4C illustrates a

typical example. Percolated islands and channel-type Au(111)-pATP patterns are obtained after longer assembly times (> 1.0 h). The estimated corrugation height of 0.2 to 0.4 nm is rather similar to the one of the protein, and therefore, no unambiguous and reliable estimation of the CueO coverage is possible, despite the fact that the current response upon polarization in oxygen saturated electrolyte clearly demonstrates the presence of catalytically active CueO.

CueO immobilization on Au(111)-CYS, which represents a densely packed, positively charged SAM, is more favorable (Figure 4D). Adsorbed CueO molecules appear in oxygen-saturated electrolyte as evenly distributed bright spots. An average coverage of 35 to 40 % could be achieved routinely (1.8 pmol cm^{-2}). Complementary experiments were carried out under anaerobic conditions, with the electrolyte carefully purged with argon and the entire STM stage positioned in an argon-filled chamber. Figure 4E illustrates a typical result, as exemplified for CueO on Au(111)-CYS at -0.10 V. The STM-image shows high-quality resolved step edges of the substrate and etch pits within the CYS adlayer in high quality. However, no bright spots, which could indicate the presence of immobilized CueO, were found. The typical bright spots occurred again when adding pure oxygen into the environmental chamber and keeping the electrochemical STM cell polarized under otherwise identical conditions (Figure 4D). These bright spots indicate unambiguously the presence of CueO on Au(111)-CYS. The appearance of this contrast pattern is typical for the entire surface. The modulation of the STM contrast pattern of CueO could be changed reversibly upon purging the system again with argon and removing the dissolved oxygen from the electrolyte. The latter process is rather slow and requires sequential argon purging cycles. A similar change in contrast pattern of CueO in the presence and in the absence of oxygen was also found for Au(111)-MPA and Au(111)-pATP.

Figure 4: (A) Cyclic voltammograms of ORR recorded in the STM cell for Au(111), and Au(111) modified with monolayers of MPA, CYS and pATP with immobilized CueO in O_2 -saturated phosphate buffer, pH = 6.5. (B) In-situ STM image of CueO on Au(111)-MPA at -0.10 V, O_2 saturated. (C) STM image of CueO on Au(111)-pATP at -0.10 V, O_2 saturated. (D) STM image of Au(111)-CYS at -0.10 V, O_2 -saturated. (E) STM image of CueO on Au(111)-CYS at -0.10 V, in argon. (F) Apparent contrast height of CueO on Au(111)-CYS in O_2 -saturated phosphate buffer, pH = 6.5, in dependence on the substrate potentials, $E_{\text{bias}} = E_{\text{T}} - E_{\text{S}} = 0.10 \text{ V}$. The inset shows a typical line cross section. The typical imaging conditions are: bias = 100 mV, tunneling current = 50 pA for (B-F).

Since the STM contrast pattern is determined by morphological and electronic properties of the tunnel junction,⁴⁶ we conclude that the “invisibility” of CueO in an oxygen-free electrochemical environment and the appearance of bright spots in the STM contrast pattern upon oxygen exposure, under otherwise identical experimental conditions, represent major electronic structure changes of the protein. The bright, protein-related contrast spots demonstrate a higher electronic transmission through CueO in the STM nanogap in the presence of oxygen. A similar observation was reported before for a related protein.²⁵ Interestingly, the apparent contrast pattern of CueO is rather independent on the applied electrode potential in $-0.15\text{ V} < E_s \leq 0.60\text{ V}$ for a wide range of bias voltages (Figure 4F). This observation is distinctly different from STM-images of other surface-immobilized redox proteins, such as azurin,⁴⁷ streptomices laccase²⁵ and hydrogenases.⁴⁸ In these three studies, the apparent STM contrast shows a well-resolved potential dependence with a maximum close to the formal potential of the proteins, which leads to the opening of an additional (resonance) tunneling channel.²⁵ Such a mechanism seems to not be dominating for the present system CueO on Au(111)-SAM. We hypothesize that the presence of oxygen leads to a major chemical structure and/or orientation change of the enzyme, which reduces the tunneling barrier and dominates possible contributions from a potential-controlled modulation of the redox-state of the CueO. This proposal shall be investigated next by surface-enhanced Raman spectroscopy with CueO on Au(111)-SAMs under electrocatalytic operating conditions.

Spectroscopic measurements

UV-Vis spectro-electrochemical experiments of CueO in phosphate buffer in the presence of 1 mM $\text{K}_4\text{Fe}(\text{CN})_6$ and 1 mM $\text{K}_4\text{W}(\text{CN})_8$ as redox mediators and employing a gold mesh working electrode (for details see experimental part and ref.⁴⁹) revealed a strong absorption band with a

maximum at 610 nm (Figure 5A). This feature is assigned to the charge transfer band $\text{Cys(S)}_{\pi} \rightarrow \text{Cu(II)}$ of the Type 1 copper site.^{50,51} The intensity of the absorption band decreases significantly upon reduction of Cu(II) to Cu(I) and is therefore a marker of the redox state of the Type 1 site. (The complete UV-Vis spectrum obtained in a standard 1 cm path length cuvette is shown in SI, Figure S2).

Complementary “bulk” Raman experiments recorded in the same spectro-electrochemical thin layer cell with a HeNe laser showed two characteristic CueO-related bands around $\sim 408 \text{ cm}^{-1}$ and $\sim 421 \text{ cm}^{-1}$ at positive potentials (Figure 5B). Similar, as the electronic transition in the UV-Vis spectra, the intensities of both Raman bands decreases upon reduction of CueO, and may therefore be considered as signatures of the redox-state of the Type 1 copper site. Figure 5B demonstrates that the center positions of the main Raman bands are rather independent of the applied electrode potential. We assign both Raman modes to vibrations of the Cu-S(Cys) bond based on reports of Nestor et al.⁵⁰ and Hildebrandt et al.⁵¹ We also note that the electronic transition with its maximum around 610 nm is in resonance with the wavelength of the excitation laser, which leads to an enhanced resonance Raman signal (RR). Exploiting the resonance between the excitation wavelength and the two Type 1 copper site related vibrations, we designed the following surface enhanced Raman experiments (SERS): The gold surface was first modified with citrate-stabilized gold nanoparticles of ca. 50 nm in diameter (ca. 20% of the electrode area were covered), which act as plasmonic antennas.⁵² Then, the SAM was created as described above by immersing the modified gold surface on the thiol solution. Finally, CueO was immobilized following the same strategy as for the voltammetric experiments. Electrochemical experiments revealed that this protocol leads for all three SAMs to electrocatalytically active electrodes for the ORR in case of all three SAMs. The surface coverage of CueO shows the same

trends in the presence as well as in the absence of nanoparticles. The highest catalytic activity was found for Au-pATP, similar as for the nanoparticle-free surface.

Figure 5: (A) UV-VIS spectra recorded for the CueO in O₂-saturated phosphate buffer in a spectro-electrochemical thin layer cell (gold grid as working electrode) in the presence of Fe(CN)₆⁴⁻/W(CN)₈⁴⁻ as redox mediator. (B) Raman spectra as recorded in the thin layer cell for the system under (A). Integrated intensities of the prominent UV/VIS band (diamonds) and of the Raman intensities for bulk protein in the thin layer cell (triangles) and for the immobilized protein (circles), in dependence on the electrode potential. Closed and open circles correspond to negative and positive sweep direction.

Figure 6: Potential-dependent surface enhanced Raman spectra for CueO immobilized on Au-CYS in phosphate buffer, pH = 6.5 (A) in oxygen saturated electrolyte and (B) in argon under unareobic conditions. (C) Potential-dependence of the integrated intensity of the Cu-S(Cys) mode ($\sim 414\text{ cm}^{-1}$) in oxygen-saturated (triangles) and argon-saturated (circles) electrolyte; closed and open circles corresponds to the negative and positive sweep directions, respectively.

Electrochemical SERS spectra were recorded after transferring the modified electrode into the custom-made Raman cell.⁵³ Gold electrodes modified with MPA, CYS and pATP showed two distinct Raman signatures around 400 cm^{-1} (Figure 6 and SI, Figures S6 and S7), which were assigned, based on bulk solution Raman experiments (Figure 5 and ref.^{50,51}), to vibrations of the Cu-S(Cys) bond in the Type 1 copper site. The potential-dependent SERS experiments were carried out under controlled reaction conditions, e.g. in an oxygen-saturated atmosphere as well as in an anaerobic argon atmosphere.

As an example, we describe next the spectra of Au-CYS in details. Similar results were obtained for Au-MPA and Au-pATP. These experiments are summarized in the SI, Figures S6 and S7. We like to note that the CueO-related bands are distorted for the latter due to interference with the strong SERS signal of pATP^{54,55}, which hampers a reliable quantitative analysis of this data.

Figure 6A displays the potential-dependent evolution of the Raman spectra for CueO on Au-CYS during the ORR in an oxygen-saturated electrolyte (phosphate buffer, pH ~ 6.5). The experiments started at 0.40 V. CueO resides in its fully oxidized state under those conditions. The corresponding Raman spectrum shows the characteristic CueO-related bands^{50,51} at 385 cm⁻¹ and 414 cm⁻¹, and two additional bands at 300 cm⁻¹ and ca. 250 cm⁻¹. The broad feature around 250 cm⁻¹ shows a slight blue shift with more negative potentials, and seems to be related to the presence of oxygen since it is not observed for the same system in argon atmosphere. The band at 300 cm⁻¹, which is also blue-shifted and increases in intensity with more negative potentials, is assigned to the Au-sulfur bond in the CYS-SAM, as one can conclude from control experiments for the Au-SAM system with gold nanoparticles as plasmonic antennas. Additional support for this interpretation comes from a comparison with literature data.^{56,57} The above CueO-free control experiments demonstrate further that no interfering spectroscopic signatures of the citrate shell could be detected in the wavenumber range 200 cm⁻¹ < ν < 600 cm⁻¹.

The intensities of the two CueO-related bands, which are both assigned to vibrations of the Cu-S(Cys) bond^{50,51} are maximum at 0.40 V (i.e. when the protein is in its fully oxidized state), and significantly decrease as the potential is reduced, although they do not disappear completely in the presence of oxygen (Figure 6C). The excursion to negative potentials and the presence of oxygen lead to changes in the redox state of the protein centers as triggered by the initialized

catalytic cycle. We also found that the position of the protein bands are not changing with potential, which might be related to the fact that the redox-active Type 1 copper center is quite far from the electrode. In consequence, it experiences rather little of the interfacial potential drop (from the 3D structure of the protein, the closest distance between the type 1 copper center and the surface is around 1.2-1.5 nm³⁰). Finally we note that reversing the potential scan towards positive values leads to a complete recovery of the initial spectroscopic response at 0.40 V (Figure 6C).

Complementary Raman experiments with the same protein-modified electrode under anaerobic conditions in a pure argon atmosphere revealed the same CueO-related bands around 400 cm⁻¹ and the Au-sulfur band at 300 cm⁻¹ as under oxygen. The protein band reaches its maximum at E = 0.40 V, and decreases upon potential excursion in the potential region of the ORR. However, a distinct difference is observed. The Cu-S(Cys) bands disappear completely when the potential is decreased below 0.2 V, while a remnant band was always visible, even at 0.00 V with O₂ being present in the solution. Changing the potential back to 0.40 V, also under argon, leads to completely recovered protein bands at 0.40 V. This behavior indicates that the process is reversible and no irreversible changes occurred with the protein during potential cycling. Figure 6C summarizes the potential dependencies of integrated intensities and wave number of the main CueO band around 414 cm⁻¹ for the negative and the corresponding positive scan in the presence as well as in the absence of oxygen. The S-shape agrees perfectly with the corresponding data extracted from potential-dependent UV-Vis data of the charge transfer band Cys(S)_π→Cu(II) at 610 nm (Figure 5C).

Similar results were obtained in Raman experiments with CueO on Au-MPA and Au-pATP. These data are summarized in SI.

DISCUSSION

The voltammetric results described above demonstrate that CueO can be immobilized on bare Au(111) surfaces by immersing a clean electrode into a protein containing solution. The protein retains its O₂ reduction activity and exhibits good electronic communication with the electrode in the absence of electron mediators. Employing self-assembled monolayers as redox mediators leads to a significantly higher catalytic activity and stability of the surface-immobilized CueO. Electrochemical screening experiments with a wide range of mediators demonstrated that NH₂-endcapped SAMs led to a particularly high ORR activity. Excellent results were obtained with CYS and pATP. Carboxylate-terminated adlayers, such as MPA, gave a much lower activity. Long alkyl chains completely quenched the activity.

Two aspects of the results presented above deserve further discussion. First, the strong change in electron tunneling through the protein as induced by the presence of O₂. Secondly, the implication of the spectroscopic results to the route of electron transfer through the protein from the electrode surface to the O₂ as final electron acceptor.

In-situ STM experiments revealed the “invisibility” of immobilized CueO in an oxygen-free electrochemical environment and the appearance of (“visible”) bright spots in the STM contrast pattern upon oxygen exposure under otherwise identical conditions. A similar effect was reported before for the related enzyme *Streptomyces* Laccase.²⁵ The exact mechanism of this effect is still unclear. However, we can hypothesize that the presence of oxygen causes major changes in the electronic and conformational structure of the protein facilitating tunneling through the enzyme. Structure changes in the enzyme during the catalytic cycle have been investigated with stop flow spectroscopic techniques, X-ray absorption spectroscopy, electron paramagnetic resonance and circular dichroism.^{36,37} The existence of two different intermediates

formed upon oxidation of the enzyme in the presence of oxygen is well established in the literature.^{16,58}

Starting from the fully reduced enzyme, with the four copper atoms in the valence state (I), the attachment of oxygen leads to the formation of a peroxide intermediate that decays very rapidly to the native intermediate (NI). While it was proposed in the past that the Type 2 Cu site of the NI was in the reduced state, more recent data shows that the NI contains four copper atoms in the oxidized state.¹⁶ The fully oxidized resting state of the enzyme also contains 4 Cu(II) ions. However, both states differ in key structural properties. The Cu(II) ions are antiferromagnetically coupled through a bridging hydroxide ligand in the resting oxidized state of the Type 3 Cu site. A second water or hydroxyl species is bound to the Type 2 Cu(II) site. However, this site is not coupled to the other Type 3 ions of the reaction center. The orientation of this water or OH species is directed opposite from the interior of the trimer. On the other hand, in the NI one or two oxygen atoms are bridging the trimer pointing towards the interior of the trinuclear cluster and coupling electronically the Type 2 to the Type 3 dimer. Such structure difference between both oxidized states make the transformation between the NI and the fully oxidized resting state structurally very demanding. Therefore, it has been proposed that the catalytic cycle restarts from the NI instead of evolving first to the fully oxidized state⁵⁸, by consumption of four electrons from the substrate or the electrode. The effect of O₂ on the rate of intramolecular electron transfer was pointed out before.^{16,59,60} The rate of intramolecular electron transfer of the fully oxidized resting state is very low as compared to the overall turnover rate of the enzyme. However, the rate of intramolecular ET has been reported to increase for the NI as a consequence of the coupling between the three copper atoms in the Type 2/3 trinuclear centre. Reduction of the Type 2 Cu(II) site is very slow for the resting state, because this centre is buried and

electronically isolated. The formation of a μ_3 -oxo bridge in the trinuclear cluster of the NI enhances the electronic coupling between Type 2 and Type 3 Cu ions, which provides new superexchange pathways for the rapid intramolecular electron transfer between the Type 1 and the Type 2/3 trinuclear centers. In consequence, the reduction from NI to the resting reduced enzyme is facilitated.^{16,59,60} The enhancement of the rate of intramolecular ET is likely to be reflected in the rate of tunneling. Climent et al. proposed for a related laccase a hopping mechanism for electrons to tunnel from a SAM-modified metal electrode to the tip of an STM through the different copper centers.²⁵

An important difference between the current study and the previous work by Climent et al. with *Streptomyces Lacasse*²⁵ is the absence of a dependence of the tunneling current on the electrode potential in the present case. The arguments in the previous paragraph suggest that the change in the valence state of Cu ions induced by the variation of the potential should modify the tunneling of electrons through the immobilized enzyme and the respective SAM. However, the absence of a potential-modulated tunneling current in the region of the CueO-catalyzed oxygen reduction suggests that the presence of oxygen to bridge the trinuclear center is the key factor for the rate of tunneling while the valence state of the Type 1 Cu center is less important for electron transport through the entire nanoscale bridge.

Figure 7: Catalytic cycle for the CueO-mediated ORR with a representation of the reaction sites Type 1 to Type 3 as derived from the present experimental data, which support certain steps and pathways.

In an attempt to explore structure changes at and around the Type 1 copper center during the ORR and in an argon atmosphere upon polarization, we carried out SERS experiments with gold nanoparticles as plasmonic antennas. The charge transfer band $Cys(S^-)_\pi \rightarrow Cu(II)$ of the Type 1 copper center with a maximum in the UV-Vis spectrum at 610 nm leads to detectable resonance Raman signals of the Cu-S (Cys) bond at $\sim 385\text{ cm}^{-1}$ and $\sim 414\text{ cm}^{-1}$ upon excitation with a HeNe laser (Figure 5 and Figure 6). Both signals are quantitative markers of the redox state of the Type 1 copper site. Trends of the potential-dependent normalized Raman intensity are identical with those extracted from UV-Vis spectra³² (Figure 5C) and lead nearly to the same standard potential of the Type 1 copper centre. The estimated value amounts to $\sim 0.260\text{ V}$. Previous reports

demonstrated the existence of a direct electronic communication between the electrode and the Type 1 copper center for CueO in solution.³² However, our current study represents, to the best of our knowledge, the first report on the redox state of the Type 1 copper centre to be determined for the immobilized enzyme on the electrode surface and under reaction conditions. The reduction of the Type 1 Cu from Cu(II) to Cu(I) as the potential is lowered is a clear indication of the good electronic communication between this redox center in the immobilized enzyme and the electrode surface. Moreover, the comparison of measurements in oxygen- and argon-containing environments provide important clues about the participation of this redox center in the catalytic cycle of the enzyme. While the Raman resonant bands completely disappear in an argon atmosphere, measurements under oxygen always resulted in persisting CueO-related bands at 385 cm⁻¹ and 414 cm⁻¹ (Figure 6C). This observation implies that a small but distinct portion of Cu(II) exists in the catalytic cycle, even at the most negative potentials investigated. This agrees with a mechanism in which electrons enter the protein through the Type 1 copper center and are then transferred to the Type 2/3 copper center to be used in the oxygen reduction process. In the absence of oxygen, Cu(I) remains in the reduced state. However, if oxygen is present in solution, Cu(I) is reoxidized to Cu(II) as the electrons are used in the conversion of oxygen to water.

The same trend is observed with the enzyme immobilized on a NH₂- or COOH-terminated SAMs, and even on the bare gold surface (see SI). However, the intensities of the bands are greater with the NH₂ - modified surface suggesting that the orientation in this case results in the Type 1 copper center closer to the surface. Nevertheless, in all cases studied the entry point of electrons to the enzyme seems to be the Type 1 copper center since the complete reduction of

Type 1 Cu(II) to Cu(I) depends on the absence of oxygen that otherwise reoxidizes the Cu(I) to Cu(II).

In summary, SERS experiments with plasmonic nanoparticles suggest a significant decrease in the population of Type 1 Cu(II) sites with more negative potentials in the catalytic wave. However, STM measurements indicate that this decrease has no implications on the rate of tunneling through the enzyme. This discrepancy points to electronic structure changes of the reaction site in the presence of oxygen. Theoretical calculations and simulations are currently being performed to address this hypothesis in more detail.

CONCLUSIONS

The catalytic activity of multi-copper oxidase CueO immobilized on gold substrates was investigated by cyclic voltammetry, surface enhanced Raman spectroscopy and scanning tunneling microscopy. Electrodes modified with self-assembled monolayers as redox-mediators showed the highest activity with NH₂-solution exposed end groups of the respective SAMs. This trend was rationalized by analyzing the composition at the surface of the enzyme. In situ STM experiments revealed that the presence of oxygen causes major changes in the electronic and conformational structure of the protein facilitating the tunneling of electrons in electrified nanoscale electrode-CueO-electrode assemblies. In particular, we observed electronically silent CueO reaction sites under anaerobic conditions in argon, and highly active CueO sites in the presence of oxygen.

Raman spectra revealed unambiguously clear redox-marker bands at $\sim 385\text{ cm}^{-1}$ and $\sim 414\text{ cm}^{-1}$ corresponding to the Cu-S(Cys) vibration in the Type 1 copper center. The integrated intensities of these bands are sensitive to the redox state of copper in the Type 1 center of CueO allowing monitoring changes in the oxidation state of the metallic center during the catalytic cycle of the

immobilized enzyme in the absence as well as in the presence of oxygen under fully operating conditions. The reduction of the Type 1 Cu from Cu(II) to Cu(I) as the potential is lowered is a clear indication of the good electronic communication between this redox center in the immobilized enzyme and the electrode surface.

EXPERIMENTAL SECTION

Cyclic voltammetry

The electrochemical experiments were performed in a conventional three-electrode configuration using an Autolab PGSTA302N (Metrohm/Ecochemie). An Au(111) hemispherical bead served as working electrode. The counter electrode was a gold wire. A reversible hydrogen electrode in a separate compartment was used as reference. The potential of the latter was measured at the end of each experiment versus an Ag/AgCl/KCl(sat) reference electrode. The potentials in this paper are referred to this scale. All experiments were performed at room temperature.

Spectroelectrochemical experiments:

The UV-VIS spectra were recorded with a Varian Cary 500 spectrophotometer in a thin layer quartz cell (BASi Inc.®) under potential control.⁴⁹ The working electrode was a gold net. The cell was further equipped with a Pt wire as counter and an Ag/AgCl/KCl(sat) electrode as reference. Argon (Alphagaz 99.999 %) was passed over the solution in the cell during all experiments. Before every experiment, the gold net was cleaned in carboxylic acid, rinsed with ultrapure water, and dried in an argon stream.

The Raman measurements were performed under potential control with a LabRAM HR800 confocal Raman microscope (Horiba Jobin Yvon) equipped with a lab-made potentiostat and employing a custom-designed Raman cell.⁵³ The excitation wavelength was 632 nm (He-Ne

laser). The power of the laser was 1 mW, the laser was focused 50 μm above the electrode surface to avoid damage of the protein upon exposure. The signal was typically accumulated for 1 s, and three independent sets of spectra collected at different positions were averaged to obtain representative data.

Surface-confined gold nanoparticles served as plasmonic antennas. We used citrate-stabilized gold nanoparticles with a mean diameter of 55 nm, which were synthesized by reducing HAuCl_4 with sodium citrate following a previously described procedure.^{61,62} A drop of the nanoparticle-containing solution was casted on the surface of a gold electrode (Au(poly) or Au(111)) and subsequently dried in a gentle stream of argon. The resulting coverage ranged between 20 to 30 %. Next, CueO was deposited from an aqueous protein solution onto the modified surface. In some cases, the surface was modified with a ω functionalized thiol SAM, as described later, by immersing the nanoparticle modified electrode in the thiol solution, prior to the deposition of the protein.

Scanning tunneling microscopy

The in-situ STM experiments were performed with a PicoScan (Molecular Imaging) and/or a Nanoscope E (Digital Instruments) scanning tunneling microscope housed in a home-build environmental glass chamber. The working electrodes were spherical Au(111) single crystal beads of 2 to 3 mm in diameter. Platinum wires served as counter and pseudo-reference electrodes, respectively. All potentials were converted into the Ag/AgCl/KCl(sat) scale. The STM tips were either electrochemically etched or mechanically cutted Pt/Ir wires coated with polyethylene to reduce Faradaic currents to a level below 5 pA. Image processing was carried out with the WSxM software package[®] or Gwyddion (<http://gwyddion.net/>).

Chemicals and Solutions

Phosphate buffered solutions of pH 6.5 were prepared using NaH_2PO_4 (ACS reagent, Sigma Aldrich) and Na_2HPO_4 (ReagentPlus, Sigma-Aldrich®) and Ultrapure water from a Millipore System (18.2 $\text{M}\Omega\cdot\text{cm}$ and < 3 ppm total organic content). Mercaptopropionic acid (MPA), cysteamine (CYS) and p-aminothiophenol (pATP) (pure analysis, Sigma-Aldrich®) were used for the formation of self-assembled monolayers (SAM) on the respective gold electrodes and served as electron transfer mediators and “organic glue”. The MPA and CYS solutions were prepared with ultrapure water. Ethanol (pure analysis, Aldrich®) solution was used for pATP.

Molecular biology techniques

Standard molecular biology techniques were performed as previously described⁶³. Oligonucleotides were from Life Technologies and restriction enzymes and Prime STAR HS DNA polymerase from Takara. The PCR product was purified with the High Pure plasmid isolation kit (Roche Applied Science). DNA fragments were purified with Gene-Clean Turbo (Q-BIO-gene). Plasmidic DNA was isolated with the QIAprep Spin Miniprep Kit (Qiagen). T4 DNA ligase was purchased from Roche Applied Science. Transformation of *E. coli* cells was carried out using the CaCl_2 method⁶³. The cloned inserts were confirmed by DNA sequencing with fluorescently labelled dideoxynucleotide terminators and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems).

Construction of pERCueO plasmid

The DNA fragment containing the *cueO* gene was amplified by PCR from plasmid pCueO using the oligonucleotides 5'-GCAGTCCCATGGCAGAACGCCCAACGTTACCGA-3' (*NcoI* restriction site underlined) and 5'-GACTACTCGAGTACCGTAAACCCTAACATCATCCC-3' (*XhoI* restriction site underlined) as primers. The PCR product was purified, digested by *NcoI*

and *Xho*I and cloned into the same restriction sites of the expression vector pET-21d(+) (Novagen) rendering the plasmid pETCueO. Integrity of the construction was checked by DNA sequencing.

Purification of CueO protein

The plasmid pETCueO codes for CueO protein containing six histidines in the C-terminus. *Escherichia coli* BL21(DE3) cells harbouring the plasmid pETCueO were grown with aeration in 1 L Luria-Bertani medium⁶³ containing 100 $\mu\text{g mL}^{-1}$ ampicillin and 1 mM CuCl_2 until the culture reached an O.D._{600nm} around 0.6. Overexpression of the cloned gene was then induced by the addition of 0.5 mM isopropyl- β -D-1-thiogalactopyranoside. The incubation proceeded overnight at 32 °C. The cells were centrifuged at 4 °C, (5000xg), resuspended in 60 mL of 50 mM sodium phosphate buffer, 300 mM NaCl, pH 7.0, disrupted by sonication (Branson Sonifier) and centrifuged again at 4 °C (10000xg). Imidazole was then added to the supernatant at a final concentration of 20 mM. The resulting solution was then added to 15 ml of nickel-chelated butyl sepharose 4 Fast Flow (GE Healthcare) previously equilibrated in the same buffer as the sample. The suspension was shaken in batch at 10 °C for 1 h at 80 rpm, and then applied to a column (10x1 cm). The resin was subsequently washed with buffer solutions containing 20 mM and 75 mM imidazole and the protein was finally eluted with 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole with a purity of more than 95% as assessed by SDS-PAGE (Figure S1).

The corresponding aliquots were stored at 4°C. Before carrying out the voltammetric measurements, the protein was dialyzed against 50 mM sodium phosphate, 100 mM NaCl. The UV-Vis spectrum of the purified protein showed the characteristic maxima at 610 nm and 330 nm, which are assigned to the Type 1 and 3 copper centers (Figure S2)¹⁵, and the protein concentration was determined spectrophotometrically using a molar absorption coefficient at 280

nm of $63000 \text{ M}^{-1}\text{cm}^{-1}$ as predicted by ProtParam software (<http://web.expasy.org/protparam>). The enzymatic activity was tested at 23°C by following the oxidation product of 2 mM of 2,6-dimethoxyphenol (DMP) by 200 nM of CueO protein in 100 mM sodium phosphate, pH 6.5. The reaction product 3,3',5,5'-tetramethoxydiphenoquinone has an absorption coefficient $\epsilon = 49600 \text{ M}^{-1} \text{ cm}^{-1}$ at 468 nm. The protein activity was typically 0.2-0.5 UA / mg (One unit of activity, UA, is the amount of protein capable of oxidizing 1 nmol of DMP in one minute).

Preparation of electrodes

The Au(111) electrodes were annealed in a butane flame for 2 min, cooled down in an inert argon atmosphere, and are subsequently quenched in ultrapure water. They were then transferred into the electrochemical cell (for experiments with the bare electrode) or immersed in 1 mM deoxygenated aqueous or ethanolic solutions of the respective thiols to form the corresponding SAMs. Assembly was performed in the absence of oxygen in closed containers at room temperature, typically for a period of 1 hour. The electrode was then carefully rinsed with water (CYS or MPA) or ethanol (pATP). Protein immobilization on both, the bare as well as the SAM-modified electrodes was carried out by immersing them into the protein solution (0.5-3 mg/ml) for 5 minutes followed by extended rinsing with buffer solution.

Exploring experiments with Au(111)-(1x1) electrodes, i.e. the $(p \times \sqrt{3})$ reconstruction as formed during flame annealing⁶⁴ was lifted by polarization at positive charge densities in aqueous phosphate buffer solution in the presence of small concentrations of chloride ion ($< 1 \text{ mM}$), did not show major changes in surface structure and electrocatalytic response under our experimental conditions. However, all experiments with the bare electrode surface were carried out with Au(111)-(1x1) substrates to simplify the initial surface morphology and to avoid induced

contributions from additional defects sites (gold islands from the lifting of the $(p \times \sqrt{3})$ reconstruction).

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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SUPPORTING INFORMATION AVAILABLE

Purification of CueO protein by SDS-PAGE. UV-vis absorbance spectrum of CueO. Time evolution of the catalytic response for CueO immobilized on bare Au(111). Time evolution of the surface morphology of pATP monolayer as measured with STM. Cyclic voltammetry of CueO immobilized on a CYS modified Au(111) surface, as measured in the STM cell. SERS of CueO immobilized on a MPA pATP modified Au surfaces. Rotating ring – disk experiments to detect H₂O₂ formation. Effect of Cu²⁺ presence on the electrocatalysis of ORR. This information is available free of charge via the Internet at <http://pubs.acs.org>.

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