An SECM study on the influence of cationic, membrane-active peptides on a gold-supported self-assembled monolayer

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Abstract

The influence of membrane active peptides, Tat_{44-57} (activator in HIV-1) and melittin (active content of bee venom), on self- assembled monolayers of 6-mercaptohexanoic acid (MHA) on gold electrodes has been studied with scanning electrochemical microscopy (SECM). It was found that MHA, when deprotonated at physiological pH, significantly affected the relative rates of electron transfer between between the $[Fe(CN)_6]^{4-}$ solution based mediator and the underlying gold electrode, predominantly by the electrostatic interaction between the mediator and MHA. Upon the introduction of Tat_{44-57} or melittin to the electrolyte, the relative rate of electron transfer through the MHA layer could be increased or decreased depending on

the mediator used. However, in all cases it was found that these peptides have the ability to be incorporated into synthetic SAMs, which has implications for future electrochemical studies carried out using cell mimicking membranes immobilised on such layers.

1. Introduction

The modification of electrode surfaces with self-assembled monolayers (SAMs) has been widely researched. Of relevance here is that they are routinely used in the first step to fabricate a biocompatible surface that can subsequently be modified and used for biosensing, studying electron transfer of immobilised proteins and assembling scaffolds consisting of a bilayer lipid membrane that mimic biological membranes and cells [1-7]. The most commonly employed electrode material is gold due to its biocompatibility in the bulk state and the ability to form well packed and insulating SAMs through gold-thiol linkage chemistry [8]. Numerous electrochemical studies have investigated the influence of SAM composition and chain length on the rate of electron transfer between solution based redox species and the underlying electrode by means of cyclic voltammetry and spatially localised techniques, such as scanning electrochemical microscopy (SECM) [9-15]. The latter is particularly useful as it avoids large uncompensated resistance effects in the SAM and minimises double layer charging associated with cyclic voltammetric experiments [13].

In this study we focussed our attention on two membrane active peptides; Tat_{44-57} (Tat), a cell penetrating peptide (CPP), capable of delivering intracellular cargo [7, 16] and melittin an antibacterial peptide, which is a promising class of bioactive molecules to fight the increasing number of antibiotic resistant bacteria [17]. In some cases these peptides directly act by disruption of bacterial membranes or by translocation prior to acting on intracellular targets [18]. Due to the complexity of biological systems, it is often required that fundamental modes of action are initially carried out *in vitro*, on assemblies that mimic nature [5, 6, 19-22].

In general, the first step is the immobilisation of an insulating SAM on a gold surface to facilitate the attachment of membranes, proteins or lipids that then allow for characterisation with electrochemical, spectroscopic and microscopic techniques. In particular, we investigate the influence of membrane active peptides Tat (trans-activating transcriptional activator) from the human immunodeficiency virus 1 (HIV-1) and melittin, the principle active component of bee venom, towards a SAM on evaporated gold electrodes using SECM.

2. Experimental

Absolute ethanol (≥99.7%), propan-2-ol (≥99.0%) and hydrogen peroxide (30%) were purchased from Merck. Ammonium hydroxide solution (28%) was obtained from Ajax Finechem. 6-mercaptohexanoic acid (MHA, 90%), ferrocenemethanol (FcMeOH) and potassium ferrocyanide ($K_4[Fe(CN)_6]$) were purchased from Sigma-Aldrich. Tat (Tat_{44-57}) with N-terminal acetylation and C-terminal amidation was synthesised with L-amino acids by automated solid phase peptide synthesis on a Rink amide resin. The Tat sequence was Ac-GISYGRKKRRQRRR-NH_2. The melittin was purchased (97.0% purity, GL Biochem, Shanghi), with the sequence GIGAVLKVLTTGLPALISWIKRKRQQ-NH_2 and used as provided. Gold coated QCM sensors (Q-sense) were used as substrates and cleaned in NH_4OH:H_2O_2:H_2O (1:1:3 v/v) for 20–25 min at ca. 70 °C and then rinsed with ultrapure water (Sartorius). Surface modification with MHA was conducted by immersing a freshly cleaned chip into a 1 mM solution of MHA in propan-2-ol for 24 h. Excess MHA was removed by rinsing with propan-2-ol. The SECM approach curve experiments were carried out with a CH Instruments SECM (CHI920D) at an unbiased substrate with 1.0 mM $K_4[Fe(CN)_6]$ or FcMeOH in PBS (phosphate buffered saline, 20 mM phosphate, 100 mM NaCl, pH = 6.9) as the mediator, using a 25 μm diameter Pt ultra-microelectrode (UME), Ag/AgCl (3 M NaCl) and a Pt wire as working, reference and counter electrodes, respectively. Approach curves

were fitted using a LabVIEW program (PAC_Fit.vi) [23]. XPS measurements were carried out with a Thermo K-Alpha XPS instrument with core levels aligned with the C 1s binding energy of 285 eV. QCM studies were carried out as reported previously [7].

3. Results and discussion

MHA was chosen as the SAM as carboxylic acid terminated layers are suitable for the formation of lipid bilayers via liposome deposition that mimic cell membranes [5]. At physiological pH in PBS (pH = 6.90±0.02), MHA will be deprotonated and have a negative charge. At a chain length of 6 carbon atoms it is expected that electron transfer from a solution mediator to the underlying gold electrode will still occur with a well formed alkane thiol layer [11]. However, the presence of a negatively charged carboxylic acid group is likely to affect this behaviour when a charged electroactive mediator is used in SECM experiments. Therefore, we employed FcMeOH and $[Fe(CN)_6]^{4-}$ as mediators to probe differences in coulombic interactions with the negatively charged MHA layer.

Approach curves to Au/MHA using FcMeOH as the mediator exhibit a significant positive feedback response indicating electron transfer is not significantly inhibited through MHA (Figure 1a). This is reproducible across the same and different samples with a 4.4 % standard deviation in feedback current at 0.5 *d/a* demonstrating the homogeneity of the SAM. However, a completely different situation is observed with $[Fe(CN)_6]^{4-}$ whereby a significant decrease in feedback current was observed (Figure 1b). The approach curves were fitted to an established model [23-25] to give apparent heterogeneous electron transfer rate constants (k_{app}) of 0.137 and 0.002 cm s^{-1} for FcMeOH and $[Fe(CN)_6]^{4-}$ respectively. It should be noted that these values are regarded as being a measure of apparent rates of electron transfer that can be used for comparative purposes, rather than the intrinsic heterogeneous electron transfer rate. These data are consistent with the charge on the mediator significantly impacting the

SECM approach curve response to a Au-MHA electrode (Figure 2). It appears that when $[Fe(CN)_6]^{3-}$ is electrogenerated by oxidation of $[Fe(CN)_6]^{4-}$ at the tip and diffuses to the negatively charged MHA, repulsive electrostatic forces occur which inhibit the rate of electron transfer through the SAM. In contrast, $FcMeOH^+$ generated at the tip will be attracted to the MHA layer and electron transfer will remain rapid (k_{app} = 0.137 cm s^{-1}) and give the characteristic tunnelling response seen for short chain alkane thiol SAMs [5]. This behaviour is consistent with that reported for pH sensitive UME probes that utilise 11-mercaptodecanoic acid layers in a solution containing $[Fe(CN)_6]^{4-}$ [11]. However, while it is noted that the presence of pinhole defects in the MHA monolayer, and therefore direct interaction of the mediator with the gold surface [13], cannot be totally discounted, this imperfection should contribute equally in both the FcMeOH and $[Fe(CN)_6]^{4-}$ cases.

The application of SECM to test the outcome of strong interactions of membrane penetrating Tat and melittin with the MHA layer on the micron scale, which is the main aim of this study, is based on the fact that the SAM can be utilised in the fabrication of lipid bilayers that are mimics for cell membranes [20]. When FcMeOH was used as the mediator, the introduction of Tat into the bulk PBS solution dramatically decreased the feedback current (k_{app} = 0.012 cm s^{-1}) (Figure 1a), whereas, with melittin there was only a slight decrease (k_{app} = 0.045 cm s^{-1}). This outcome indicates that the underlying conducting electrode does not become more exposed to the electrogenerated $FcMeOH^+$ ions, on addition of the peptide, as in this case, the feedback current should not decrease. Rather, an inhibiting screening effect has been introduced when the peptides are incorporated within the film. At physiological pH, Tat has a charge of +9 whereas melittin has a +5 charge or +6 if the N-terminus is protonated [5, 20, 26]. Therefore, penetration of either peptide into the MHA layer would be expected to introduce positive charges within the film that would electrostatically inhibit $FcMeOH^+$ diffusing to the surface of Au/MHA, thereby decreasing the rate of electron transfer.

Differences in feedback data suggest that the Tat peptide is incorporated to a greater extent within the MHA layer, with the higher positive charge also potentially playing a small role. Recent studies showed that electron transfer through insulating alkane thiol layers is facilitated via attachment of gold nanoparticles to SAM surface [9, 27]. This was explained via the formation of a metal/insulator/metal sandwich structure, which allowed superior efficiency of electron transfer than available for electron transfer between a metal and an electroactive species in solution [9]. However, this type of mechanism is unlikely to occur with peptides, where the change in electron transfer through MHA is likely to be governed almost exclusively by electrostatic effects.

The dominance of electrostatic interactions was further supported when $[Fe(CN)_6]^{4-}$ was used as the mediator. In this situation, introduction of Tat into solution led to a significant increase in feedback current (k_{app} = 0.005 cm s^{-1}) (Figure 1b). This outcome is consistent with positively charged Tat within the MHA layer facilitating electron transfer through the SAM via screening the negative charge on the MHA layer or by electrostatic attraction of the electrogenerated $[Fe(CN)_6]^{3-}$ or a combination of both effects. When melittin was used, the impact on feedback was again much less significant (k_{app} = 0.002 cm s^{-1}) as found when using FcMeOH as the mediator. This trend further supports the hypothesis that more Tat is incorporated within the MHA layer than melittin. These results are consistent with the "repelling mode" of SECM introduced by Schumann who observed significant decreases in positive feedback currents above DNA-modified electrodes due to repulsion between deprotonated phosphate groups of the DNA strands and the $[Fe(CN)_6]^{3/4-}$ mediator [28, 29].

The incorporation of peptide into the SAM was supported by QCM data obtained *in situ* using melittin. Analysis of this data clearly shows a mass increase (frequency decrease) upon introduction of melittin into the SAM layer at time = 30 min (Figure 1c). Further analysis of the penetration depth, using the 3^{rd}, 5^{th}, 7^{th} and 9^{th} harmonics of the QCM sensors

natural frequency (5 MHz), is consistent with the SECM analysis that incorporation of the melittin peptide into the SAM occurred rather than surface adsorption [30, 31]. Furthermore, the dissipation component of the QCM data indicates that the new layer is stiffer than the pure MHA. *Ex situ* XPS also confirms the presence of melittin via the N 1S core level peak at a binding energy of 400.1 eV as well as the presence of MHA via the S 2p doublet at 161.9 and 163.2 eV (Figure 1d). Figure 2 provides a schematic summation of changes in the interaction of the mediator with MHA/Au in the absence of peptides (Figure 2a,d) and presence of Tat (Figure 2b,e) and melittin (Figure 2c,f) and indicates the role of electrostatic interactions between the mediators and the charge on the surface. It should be noted that Figure 2 is not drawn to scale and solely illustrates the mechanism of mediator recycling that occurs over several domains of the SAM.

4. Conclusions

Studies described in this paper suggest that electrostatic interactions between a solution mediator and an electrode consisting of a SAM of MHA on gold dominate the apparent rates of electron transfer through the MHA layer. This was evidenced by changes in the feedback current in SECM approach curves to the negatively charged MHA with FcMeOH and $[Fe(CN)_6]^{4-}$ as mediators, where apparent electron transfer rates were unaltered and diminished, respectively. Significantly, membrane interacting peptides, such as Tat and melittin interact substantially with SAMs of MHA on gold. Using SECM approach curves, QCM and XPS data we showed that these peptides possess the ability to penetrate well adhered SAMs, Tat more so than melittin, which enables electron transfer from a solution mediator to the underlying gold surface to be either increased or decreased due to alteration of electrostatic interactions between the mediator and peptide within the SAM. It is believed that

this result provides an important insight into how exceptional penetrating ability of membrane active peptides may be used to modulate the characteristics of chemically modified electrodes.

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References

[1] J.J. Gooding, F. Mearns, W. Yang, J. Liu, Electroanalysis, 15 (2003) 81.

[2] X. Zhang, X. Du, X. Huang, Z. Lv, J. Am. Chem. Soc., 135 (2013) 9248.

[3] J.J. Gooding, S.G. Parker, Y. Lu, K. Gaus, Langmuir, 30 (2013) 3290.

[4] F.A. Armstrong, H.A.O. Hill, N.J. Walton, Acc. Chem. Res., 21 (1988) 407.

[5] S. Piantavigna, G.A. McCubbin, S. Boehnke, B. Graham, L. Spiccia, L.L. Martin, Biochim. Biophys. Acta, 1808 (2011) 1811.

[6] J. Kim, A. Izadyar, N. Nioradze, S. Amemiya, J. Am. Chem. Soc., 135 (2013) 2321.

[7] S. Piantavigna, M.E. Abdelhamid, C. Zhao, X. Qu, G.A. McCubbin, B. Graham, L. Spiccia, A.P. O'Mullane, L.L. Martin, ChemPlusChem, (2014) DOI: 10.1002/cplu.201402209.

[8] L. Newton, T. Slater, N. Clark, A. Vijayaraghavan, J. Mater. Chem. C, 1 (2013) 376.

[9] J.B. Shein, L.M.H. Lai, P.K. Eggers, M.N. Paddon-Row, J.J. Gooding, Langmuir, 25 (2009) 11121.

[10] S. Amemiya, A.J. Bard, F.-R.F. Fan, M.V. Mirkin, P.R. Unwin, Annu. Rev. Anal. Chem., 1 (2008) 95-131.

[11] F.-M. Boldt, N. Baltes, K. Borgwarth, J. Heinze, Surf. Sci., 597 (2005) 51.

[12] F. Forouzan, A.J. Bard, M.V. Mirkin, Israel J. Chem., 37 (1997) 155.

[13] B. Liu, A.J. Bard, M.V. Mirkin, S.E. Creager, J. Am. Chem. Soc., 126 (2004) 1485.

[14] C. Cannes, F. Kanoufi, A.J. Bard, J. Electroanal. Chem., 547 (2003) 83.

[15] A. Kiani, M.A. Alpuche-Aviles, P.K. Eggers, M. Jones, J.J. Gooding, M.N. Paddon-Row, A.J. Bard, Langmuir, 24 (2008) 2841.

[16] A.T. Jones, E.J. Sayers, J. Controlled Release, 161 (2012) 582.

[17] M. Zasloff, Nature, 415 (2002) 389-395.
[18] P. Czihal, D. Knappe, S. Fritsche, M. Zahn, N. Berthold, S. Piantavigna, U. Müller, S. Van Dorpe, N. Herth, A. Binas, G. Köhler, B. De Spiegeleer, L.L. Martin, O. Nolte, N. Sträter, G. Alber, R. Hoffmann, ACS Chem. Biol., 7 (2012) 1281.
[19] G.A. McCubbin, S. Praporski, S. Piantavigna, D. Knappe, R. Hoffmann, J.H. Bowie, F. Separovic, L.L. Martin, Eur. Biophys. J. Biophys. Lett., 40 (2011) 437.
[20] S. Piantavigna, M. Abdelhamid, C. Zhao, X. Qu, B. Graham, L. Spiccia, A. O'Mullane, L.L. Martin, J. Peptide Sci., 18 (2012) S80.
[21] S. Piantavigna, P. Czihal, A. Mechler, M. Richter, R. Hoffmann, L. Martin, Internat. J. Peptide Res. Therapeutics, 15 (2009) 139.
[22] P. Sherman, R. Jackway, J. Gehman, S. Praporski, G. Mccubbin, A. Mechler, L. Martin, F. Separovic, J. Bowie, Biochemistry, 48 (2009) 11892.
[23] M. Jensen, D. Tallman, J Solid State Electrochem, 17 (2013) 2999.
[24] R. Cornut, C. Lefrou, J. Electroanal. Chem., 621 (2008) 178.
[25] C. Wei, A.J. Bard, M.V. Mirkin, J. Phys. Chem., 99 (1995) 16033.
[26] B. Bechinger, Biochim. Biophys. Acta, 1462 (1999) 157.
[27] P. Allongue, F. Maroun, Curr. Opin. Solid State Mater. Sci., 10 (2006) 173.
[28] F. Turcu, A. Schulte, G. Hartwich, W. Schuhmann, Biosens.Bioelectron., 20 (2004) 925.
[29] F. Turcu, A. Schulte, G. Hartwich, W. Schuhmann, Angew. Chem. Int. Ed., 43 (2004) 3482.
[30] A. Mechler, S. Praporski, K. Atmuri, M. Boland, F. Separovic, L. L. Martin, Biophysical Journal, 93 (2007) 3907.
[31] K. F. Wang, R. Nagarajan, C. M. Mello, T. A. Camesano, J. Phys. Chem. B, 2011, 115, 15228.

Figure 1: SECM approach curves (experimental and simulated) to Au/MHA using (a) 1.0 mM FcMeOH and (b) 1.0 mM $[Fe(CN)_6]^{4-}$ in PBS as a mediator, before and after exposure of the SAM to 10 µM Tat and melittin peptides. The Pt UME was held at 0.40 V vs Ag/AgCl using an approach speed of 1 µm s^{-1}; (c) QCM data (3^{rd}, 5^{th}, 7^{th} and 9^{th} harmonics of the QCM sensor,) recorded after exposure of Au/MHA to 10 µM melittin (from time = 30 min to 60 min) and (d) XPS spectra for N 1S and S 2p core level spectra recorded for Au/MHA that was exposed to 10 µM melittin.

Figure 2: Illustration (not drawn to scale) of the model of electrostatic interaction between FcMeOH, $[Fe(CN)_6]^{4-}$ and MHA and its effect on the mechanism of electron transfer through the MHA in the case where the peptide is absent (a, d) and when Tat (b, e) and melittin (c, f) are present within the SAM layer.

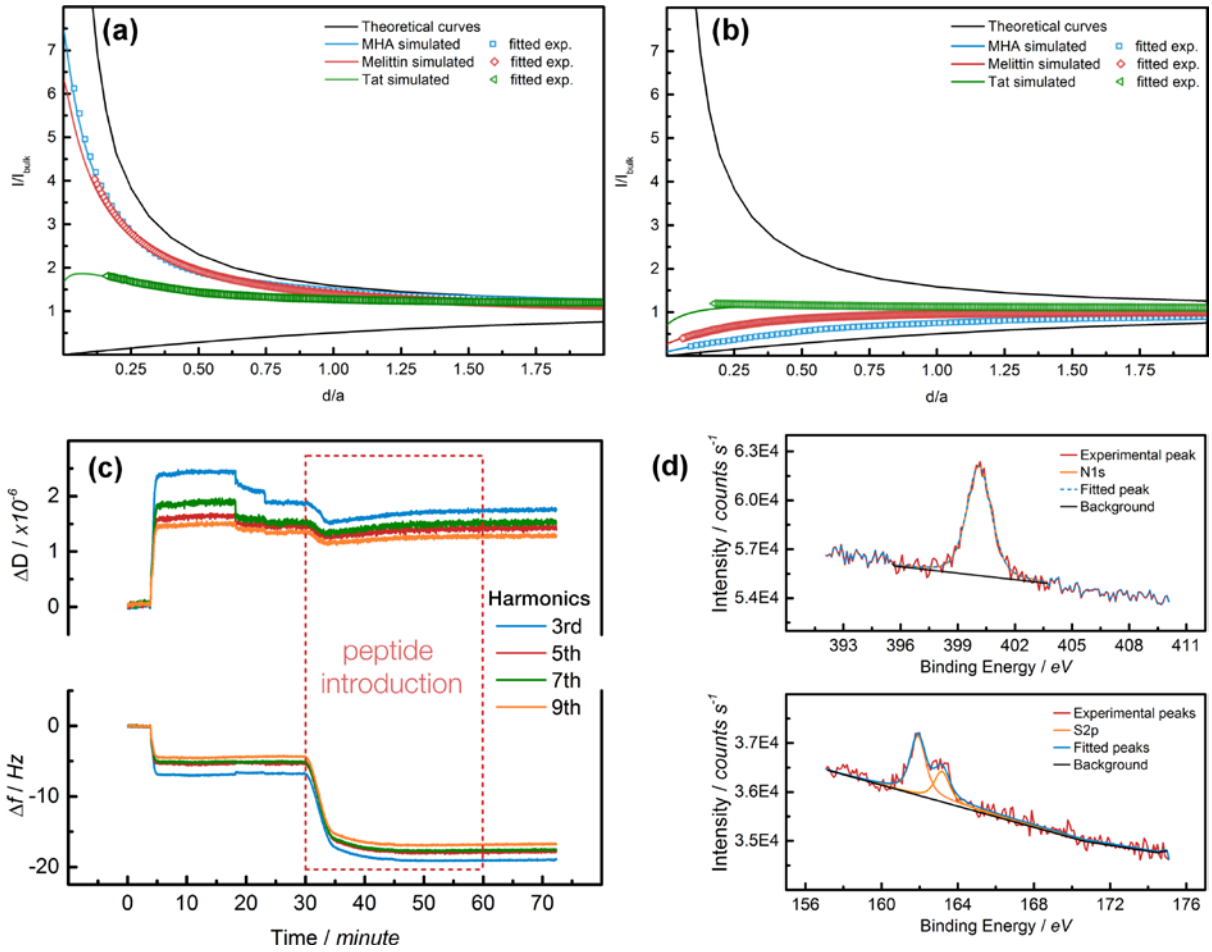
(a)
Theoretical curves
MHA simulated
Melittin simulated
Tat simulated
fitted exp.
fitted exp.
fitted exp.
I/I_bulk
d/a
(b)
Theoretical curves
MHA simulated
Melittin simulated
Tat simulated
fitted exp.
fitted exp.
fitted exp.
I/I_bulk
d/a
(c)
ΔD / x10^-6
Harmonics
3rd
5th
7th
9th
peptide introduction
Δf / Hz
Time / minute
(d)
Experimental peak
N1s
Fitted peak
Background
Intensity / counts s^-1
Binding Energy / eV
Experimental peaks
S2p
Fitted peaks
Background
Intensity / counts s^-1
Binding Energy / eV

Figure 1:

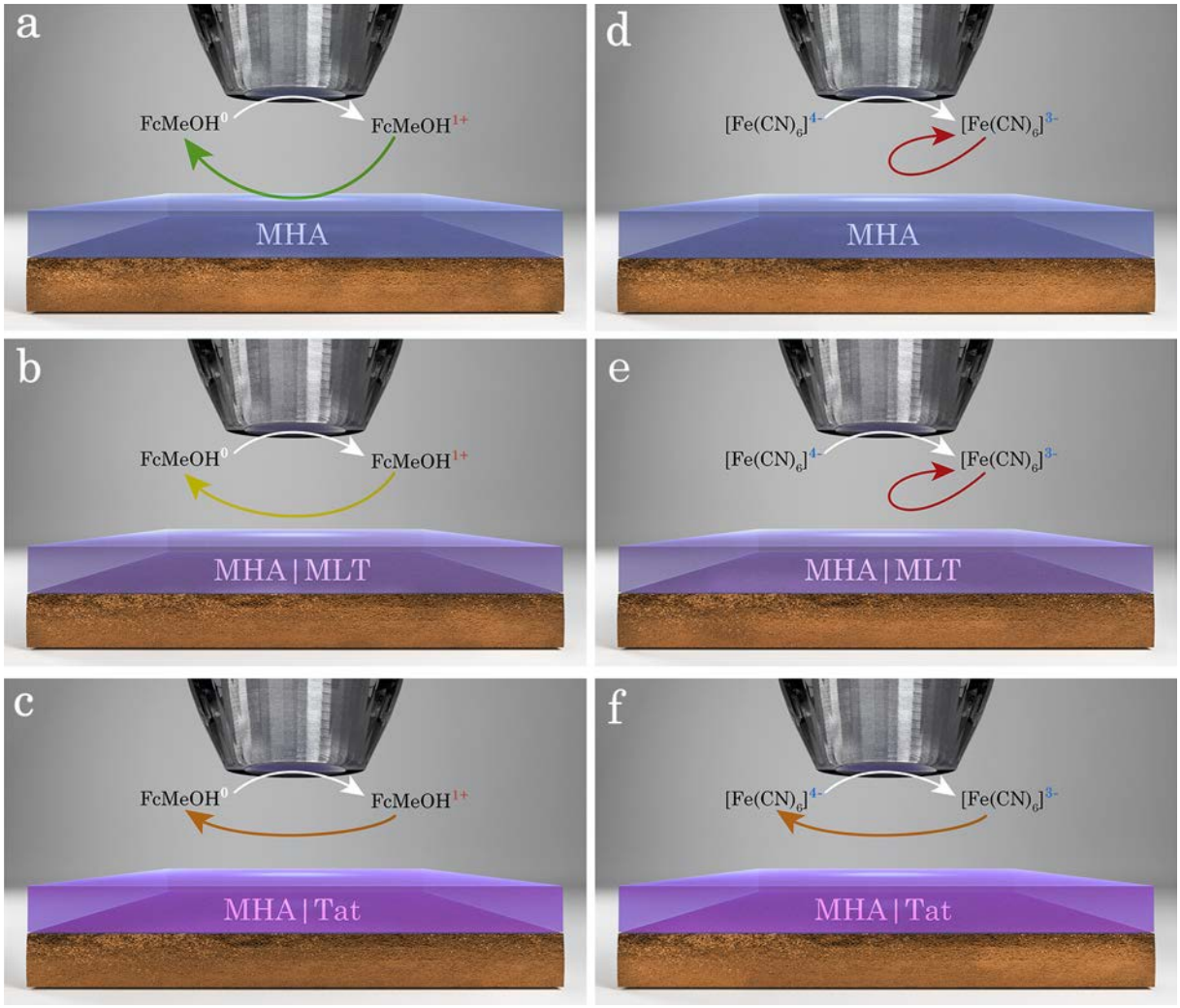
a
FcMeOH0
FcMeOH1+
MHA
d
[Fe(CN)6]4-
[Fe(CN)6]3-
MHA
b
FcMeOH0
FcMeOH1+
MHA | MLT
e
[Fe(CN)6]4-
[Fe(CN)6]3-
MHA | MLT
c
FcMeOH0
FcMeOH1+
MHA | Tat
f
[Fe(CN)6]4-
[Fe(CN)6]3-
MHA | Tat

Figure 2: