# Model systems for flavoenzyme activity: Recognition and redox modulation of flavin mononucleotide in water using nanoparticles†

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**We have used mixed monolayer protected gold clusters (MMPCs) to provide flavoenzyme model systems with a high affinity and ability to modulate cofactor reduction potential.**

Flavoproteins play a prominent role in electron transfer, oxidation, dehydrogenation, and monooxygenation reactions as well as respiration and photochemical processes.[1] They contain flavin-adenine-dinucleotide (FAD) or flavin mononucleotide (FMN) as prosthetic groups either noncovalently or covalently bound to the apoprotein.[2] These prosthetic groups enable flavoproteins to transfer electrons in a stepwise manner, either as a one electron or simultaneous two electron transfer process.[3] This property coupled with the wide variety of flavin-catalyzed transformations places flavoproteins at the crossroads of cellular redox chemistry.[4]

The redox modulation processes utilized by flavoenzymes are complex.[5] Therefore, we[6] and others[7] have developed synthetic model systems mimicking the interactions in the active site of holoenzyme between apoprotein and flavin-coenzyme. While these model studies in nonpolar media were highly informative, the challenge remains to obtain enzyme model systems providing high affinity and control over the redox chemistry of the flavin coenzyme in aqueous medium. The redox chemistry of flavoenzymes has been previously modeled using small molecules,[8] polymers,[9] proteins,[10] peptides,[11] and two-dimensional self-assembled monolayers (2D-SAMs)[12] in aprotic solvents as well as aqueous medium.

Mixed monolayer protected gold clusters (MMPCs) provide highly attractive models for flavoenzymes. MMPCs are robust, isolable, stable, portable and easy to prepare nanoscopic supramolecules.[13] They can be functionalized so that they are soluble in water as well as in organic solvents, have readily variable monolayer structures,[14] and are about the same size as enzymes. Moreover, the self-assembled monolayers of MMPCs can provide strong binding[15] through interfacial molecular recognition at their monolayer–aqueous interface. These interfaces have unique properties due to their multi-dielectric nature that differ from bulk aqueous media,[16] presenting the possibility of effective modeling of active site environments in flavoenzymes.

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Positively charged trimethylammonium functionalized gold nanoparticles (**TMA-NP**) were synthesized to provide complementary surfaces for anionic flavin mononucleotide (FMN) (Fig. 1). MMPCs were obtained by place exchanging trimethylammonium featuring alkane thiols into a gold colloid functionalized with octane thiols.[17a] The monolayer composition as characterized by $^1$H NMR featured 40% octanethiol and 60% trimethylammonium thiol functionality. The gold cores of MMPCs were *ca.* 2 nm in diameter and the overall size of particles ~6 nm. As a control, we also synthesized negatively charged mercapto-undecanoic acid functionalized gold nanoparticles (**MUA-NP**) using similar methods.[17b]

Fluorescence titrations were performed at constant pH to quantify the binding between FMN and **TMA-NP**, using the efficient quenching of fluorophores by the gold core.[18] FMN demonstrated a dramatic decrease in fluorescence intensity upon addition of **TMA-NP** (Fig. 2). The titration with noninteracting **MUA-NP** resulted in a linear decrease in the fluorescence intensity of FMN. In addition, FMN was titrated with both **MUA-NP** and

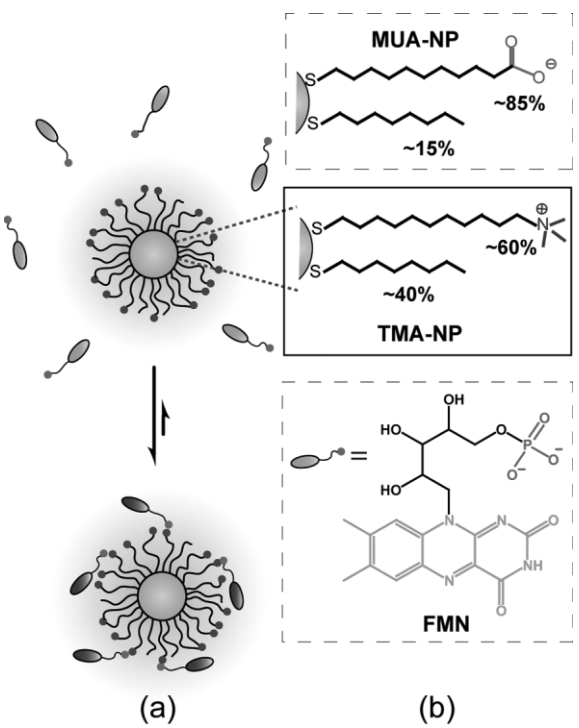


**Fig. 1** a) Schematic representation of FMN molecules binding to **TMA-NP** gold nanoparticles. b) Structures of FMN, **TMA-NP**, and control particles **MUA-NP**.

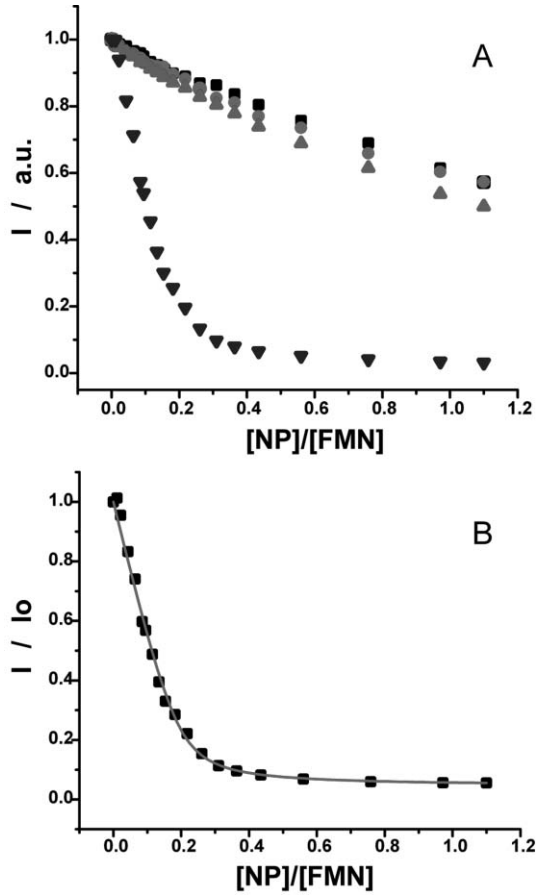


**Fig. 2** A) Fluorescence quenching curve of FMN in the presence of increasing amounts of nanoparticles at pH = 8 in water, at 23 °C. (▼) FMN titrated with **TMA-NP**, (▲) FMN titrated with **MUA-NP**, (●) FMN titrated with **MUA-NP** in 100 mM NaCl, (■)FMN titrated with **TMA-NP** in 100 mM NaCl. B) The binding curve of FMN with **TMA-NP** fitted to a binding isotherm assuming *n* identical **TMA-NP** binding sites. Data are normalized to FMN and **TMA-NP** in the presence of NaCl.

**TMA-NP** at elevated salt concentrations to account for the background quenching from the gold core. These titrations displayed similar binding curves to the **MUA-NP** alone, suggesting that the complexation between **TMA-NP** and FMN is based on electrostatic interactions.

The fluorescence titrations were normalized and fit to a binding isotherm, assuming one nanoparticle has *n* identical and independent binding sites that are able to bind one FMN molecule.[19] The microscopic association constant between one FMN and one **TMA-NP** binding site was calculated as $K = 3.7 \times 10^7\ M^{-1}$ (43.2 kJ mol$^{-1}$ binding energy per FMN). This binding is analogous to the electrostatic interactions between protein and phosphate found in the majority of flavoenzymes.[20] Since we assume that each binding site on **TMA-NP** functions independently, we can calculate the stoichiometry between the entire nanoparticle and FMN.[21] Overall, five FMN molecules bind to five **TMA-NP** sites, generating a calculated stoichiometry of 5 : 1 FMN to **TMA-NP**.

The association of **TMA-NP** with FMN far exceeds the ion pairing affinity between phosphate anion and monocationic metals (1.7–2.4 $M^{-1}$) in bulk aqueous media.[22] This dramatic increase in electrostatic binding between FMN and **TMA-NP** can be rationalized in two ways. First, the multiple cationic functions on the nanoparticle surface should allow multivalent particle–FMN interactions. A second more significant source of the high affinity arises from the interfacial properties of surfaces such as **TMA-NP**. Interfacial complexes are known to have a very high stability due to the low dielectric constant of the monolayer phase that strongly affects the charge distribution in receptor groups and their complexes. This is further supported by experiments showing that association constants ($K_a$) of ion pairs rapidly increase in going to solvents of low dielectric constant.[23] Additionally, the enhancement of intermolecular binding and other properties of interfaces such as lipid–water,[24] air–water,[25] organic–water,[26] organic–inorganic,[27] SAM monolayer–aqueous[28] has been shown in the literature.

Cyclic voltammetry (CV) experiments were used to probe the redox chemistry of FMN bound to **TMA-NP** (Fig. 3). At pH = 8 in aqueous solution, FMN alone gave a single reduction and oxidation wave with a half-wave reduction potential ($E_{1/2}$) of −492 mV *versus* Ag/AgCl, consistent with previously reported values.[29] When **TMA-NP** was added to the FMN solution at constant pH, a significant positive shift in the $E_{1/2}$ was observed. The resultant half-wave reduction potential (−394 mV) is in the range of redox potentials of flavoenzymes such as microsomal NADPH-cytochrome P-450 reductase (−390 mV) and *p*-hydroxybenzoate hydroxylase (−412 mV).[30] This +98 mV positive shift corresponds to −18.9 kJ mol$^{-1}$ stabilization, which is due to the introduction of fully reduced FMN preferentially bound to the cationically charged monolayer surface of the gold nanoparticles. This redox behavior is analogous to stabilization of flavin cofactor by flavoenzymes during redox reactions.[1–7]

NaCl was added to the FMN and **TMA-NP** mixture at the same pH to disrupt the electrostatic binding between FMN molecules and **TMA-NP**. The half-wave reduction potential shifted to a more negative value of −443 mV, indicating that FMN was removed from the surface of **TMA-NP**. High salt concentrations not only break up the electrostatic interactions between **TMA-NP**

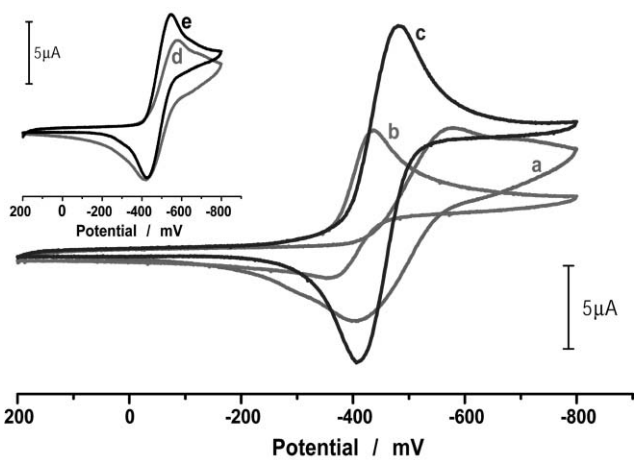


**Fig. 3** Cyclic voltammetry of (a) $5 \times 10^{-4}$ M FMN upon addition of (b) $1 \times 10^{-4}$ M **TMA-NP** and (c) 0.5 M NaCl mixed with FMN and **TMA-NP** complex at pH = 8 in water at 23 °C; scan rate, 10 mV/s *vs.* Ag/AgCl. Inset: (d) $5 \times 10^{-4}$ M FMN only and in the presence of (e) $1 \times 10^{-4}$ M **MUA-NP**.

and FMN, but also stabilize the reduced FMN (see ESI†). Therefore, the resultant FMN potential is not completely restored to its original value (−492 mV).

As a control shown in the inset in Fig. 3, the addition of negatively charged non-interacting **MUA-NP** to the FMN solution gives an $E_{1/2}$ of −485 mV, similar to the redox potential of FMN by itself. These CV experiments show that the redox chemistry of FMN immobilized on the MMPCs can be modulated and the observed redox potentials are in the range of natural flavoenzymes.

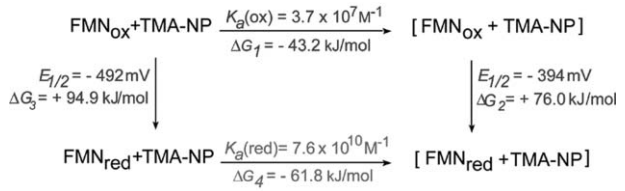


**Fig. 4** Thermodynamic cycle for the **TMA-NP** and FMN redox system.

The interdependence of redox processes and recognition events between **TMA-NP** and FMN coenzyme molecules can be quantified through use of a thermodynamic cycle (Fig. 4), where experimentally obtained $K_a$ and $E_{1/2}$ values were used to determine the $\Delta G$ and $K_a$ for binding of $FMN_{red}$ to **TMA-NP**. Using this cycle, the microscopic association constant between FMN and **TMA-NP** binding sites was found to be $7.6 \times 10^{10}$ $M^{-1}$ upon reduction of $FMN_{ox}$ to $FMN_{red}$ (see ESI†). Overall a $10^3$-fold increase in microscopic binding constant was observed, resulting in an affinity ($K_a$) of $7.6 \times 10^{10}$ $M^{-1}$ for the **TMA-NP** binding site:FMN complex.

In conclusion, we have used MMPCs to provide a highly effective model system for flavoenzymes. These particles provide high affinity binding of flavin, while the surface charge of these systems presents effective modulation of the flavin reduction potential. Efforts to integrate these attributes with specific interactions such as hydrogen bonding and aromatic stacking are currently underway.

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