

# Effect of the electrostatic microenvironment on the observed redox potential of electroactive supramolecular bioconjugates

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The present work describes our studies of the influence of the electrostatic microenvironment on the observed oxidation–reduction potential of redox-labeled supramolecular bioconjugates. Our experimental results show that the charge of the protein (streptavidin), determined by the pH, strongly influences the redox potential of the incorporated ferrocene centers. This is due to the electrostatic contributions from the fixed charges in the protein that affect the relative stability of the redox species involved in the electroactive label. In addition, the ionic strength plays a central role in screening the stabilizing or repulsive interactions, thus further influencing the magnitude of the apparent redox potential. This dependence of the redox potential on pH and ionic strength was described in quantitative terms by following a similar approach to that previously used for interpreting the behaviour of electron transfer proteins. This work provides a framework to understand, rationalize and predict variations in redox potentials of electroactive centers incorporated into different soft matter-based complex environments, like supramolecular bio-architectures. The potential of the approach was demonstrated through creation of a pH-tunable bioelectrochemical interface with switchable behavior based entirely on redox-active supramolecular bioconjugates.

## Introduction

Redox-labeled biomacromolecules are being increasingly used in different research areas, like DNA detection,<sup>1</sup> immunosensing<sup>2</sup> or even in fundamental biophysics.<sup>3</sup> The main reason for this growing interest originates from the use of electrochemistry as a simple read-out system capable of translating very specific biorecognition events into an electronic signal.<sup>4,5</sup> Many of the electrochemical strategies are based on the analysis of the electrochemical response of an electroactive label linked to the biomolecule, thus acting as a reporter of any particular biomolecular process.<sup>6</sup>

The use of electroactive labels is necessary because in most cases biomacromolecules are not intrinsically electroactive. Hence, in order to render them electroactive they are conjugated to redox centers. During the past years, a plethora of strategies involving different degrees of synthetic complexity were developed for redox-labeling diverse biomolecules with different purposes.<sup>7–44</sup>

In all the cases the redox centers are in intimate contact with the biomacromolecules. As is well known the thermodynamic characteristics of these electroactive sites are strongly affected by the nature of the environment where they are placed.<sup>45–51</sup> This is probably the reason why, just to give an example, ferrocene labels present different oxidation–reduction potentials

depending on the system where they are conjugated.<sup>52</sup> Due to its thermodynamic origin, the magnitude of the formal potential of redox centers can act as a reporter of subtle alterations in the physico-chemical characteristics of their surroundings. When dealing with biomacromolecules we need to realize that in many cases they are charged<sup>53</sup> with these charges having strong impact on the electroactive center. This has been recently demonstrated in DNA brushes by Levicky and co-workers where the formal potential of redox counterions associated with a DNA monolayer was dependent on the strands coverage.<sup>54</sup> Physically, this indicates that the ionic microenvironment of the biomolecules will potentially govern to a large extent the behavior of the redox centers bound to them.

Unraveling the role of the environmental effects in the control of redox potentials of *intrinsically* electroactive proteins has been the focus of major scientific attention during the last decades.<sup>55–62</sup> However, it should be noted that the estimation of the actual electrostatic contributions is not a trivial task.

In a similar fashion, supramolecular and bioinorganic redox chemistry<sup>63</sup> provided the platforms for creating artificial assemblies mimicking electroactive proteins.<sup>64</sup> Different strategies mostly rely on the engineering of protein-based architectures to integrate signal transduction functions, *i.e.* redox entities, directly into the assembly. One of the challenges in the molecular design of supramolecular bioassemblies is to create functional sites, like electroactive centers, into the protein environment where the surrounding matrix is responsible for the control of the reactivity of the metal center.<sup>65</sup> However, little is known about the way to predict the redox characteristics of man-made systems involving biomolecules.<sup>66</sup> In contrast to *intrinsically* electroactive proteins, the role of the

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electrostatic microenvironment in redox-active supramolecular bioassemblies (*extrinsically* electroactive proteins) has been explored to a much lesser extent.

Gaining understanding of this subject would promote significant benefits for the rational design of supramolecular functional assemblies using redox-labeled biomacromolecules as building blocks. This deeper understanding is aimed at answering questions like: What are the main factors ruling the variation of the redox potential in the supramolecular bio-assembly? Is it possible to predict the redox potential? Is it possible to think of the redox-active bioassembly as an electron transfer protein? Is it possible to reversibly tune the redox potential of supramolecular assemblies?

In this work, we focus on the critical role of the electrostatic microenvironment to tune the redox potential of ferrocene-labeled streptavidin (Fc-SAv) monolayers supramolecularly bioconjugated on biotinylated electrodes. These redox-labeled proteins could be considered as artificially generated bioinorganic hybrids with the inorganic part being in charge of the electroactive functionality and the protein itself provides the molecular recognition properties. Particularly, our aims were focused on gaining insight into why the formal potential is strongly influenced by experimental variables like ionic strength or pH. Of primary interest was to understand the influence of the ionic protein environment in such a way as to obtain a molecular level description of the factors governing the thermodynamic characteristics of the redox labels. In order to reach that goal we adapted tools previously developed to conceptualize the redox potential of well-known electron transfer proteins.<sup>67</sup> Even though the *intrinsically* electroactive electron transfer proteins differ significantly from the *extrinsically* electroactive proteins, the previous background has proven to be a suitable framework to describe man-made redox-active bioassemblies.

Our findings indicate that below the protein isoelectric point the redox potential of the ferrocene labels is shifted to anodic potentials due to the fact that the generation of the ferricinium species in a positive microenvironment is more costly. On the other hand, above the isoelectric point, the redox potential reflects a marked cathodic shift produced by the stabilization of the electrogenerated ferricinium cation in a negatively charged surrounding. Moreover, these microenvironmental changes are fully reversible enabling the fine tuning of the redox potential of the electroactive supramolecular bioconjugate over a wide range of potentials.

## Experimental procedures

### Materials

Phosphate buffered saline (PBS), *N*-hydroxysuccinimide (NHS), dimethylformamide (DMF), [*N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimide-hydrochloride] (EDC), 11-mercaptoundecanol, sodium acetate and acetic acid were purchased from Sigma-Aldrich. Streptavidin (SAv) from *Streptomyces Avidinii* was supplied by Serva (Germany). Biotin-terminated thiol [12-mercaptododecanoic-(8-biotinoylamido-3,6-dioxaoctyl)amide] was obtained from Boeringer

Mannheim. *N*-(Ferrocenylmethyl)-6-amino) hexanoic acid was synthesized as previously described by Shoham *et al.*<sup>68</sup>

### Synthesis of ferrocene-labeled streptavidin (Fc-SAv)

The redox-labeled protein was synthesized at the Max-Planck-Institut für Polymerforschung following procedures reported in the literature.<sup>69</sup> Briefly, *N*-(ferrocenylmethyl)-6-amino)hexanoic acid (36 mg), *N*-hydroxysuccinimide (14 mg) and [*N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimide-hydrochloride] (22 mg) in 1 ml of dry DMF were heated under N<sub>2</sub> atmosphere and stirring at 80 °C for 1.5 h. Nine aliquots (10–15 μl) of this solution were added to a solution constituted by 2 mg of streptavidin in 1 ml PBS buffer (0.1 M, pH 7.4). The solution was stirred overnight at room temperature with the appearance of some precipitates. The precipitates were removed by centrifugation and the supernatant was dialyzed against 0.1 M PBS buffer (pH 7.4) in order to remove any unreacted ferrocene. The spectrophotometrically determined Fc/SAv ratio was ~4. The biotin binding capacity of Fc-SAv was found to be similar to SAv.

### Electrochemical measurements

Cyclic voltammetry experiments were performed with an Autolab potentiostat using a conventional three-electrode cell equipped with an Ag/AgCl reference electrode and a graphite counter electrode.

### Biotinylation of the Au electrodes

The Au electrodes were incubated for 8–10 h in a ethanolic solution containing a binary mixture of a biotin-terminated thiol (12-mercaptododecanoic-(8-biotinoylamido-3,6-dioxaoctyl)-amide) and a hydroxy-terminated thiol (11-mercaptoundecanol) in a 1:9 ratio. This particular composition provides the optimum coverage of biotin centers for obtaining maximum streptavidin (SAv) binding, as reported by Spinke *et al.*<sup>70</sup> and López and-coworkers.<sup>71</sup>

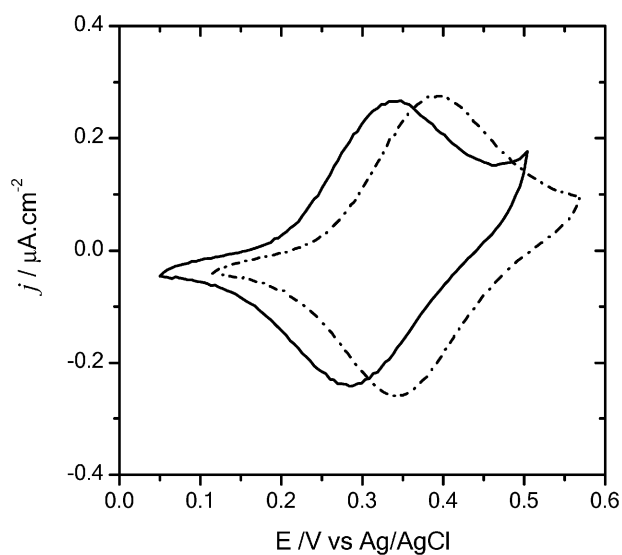
### Assembly of the redox-labeled supramolecular architecture

The bioconjugation was accomplished by placing the biotinylated electrodes in 1 μM Fc-SAv (in 0.1 M PBS) for 2 h. This led to the creation of a self-assembled interface with the redox centers lying on the protein layer.<sup>72</sup>

## Results and discussion

Fig. 1 describes the cyclic voltammetric response of the ferrocene-labeled bioconjugates immobilized on the biotinylated Au electrode in solutions containing acetate (pH 4.6) and phosphate (pH 7.4) buffers, respectively. It can be seen that changing the pH from 4.6 to 7.4 leads to a pronounced shift in the apparent formal potential of the ferrocene labels in the cathodic direction by  $\Delta E = 70$  mV.

It is well-known that ion pairing interactions can promote significant changes in the formal potential of ferrocene moieties, but this is mostly reflected in the presence of ions leading to the formation of strong ion pairs, like ClO<sub>4</sub><sup>-</sup>.<sup>73</sup> In our case, we are dealing with highly solvated anions like acetate or phosphate, that do not show the formation of strong ion pairs with ferricinium species.<sup>74</sup> Consequently, it is reasonable to

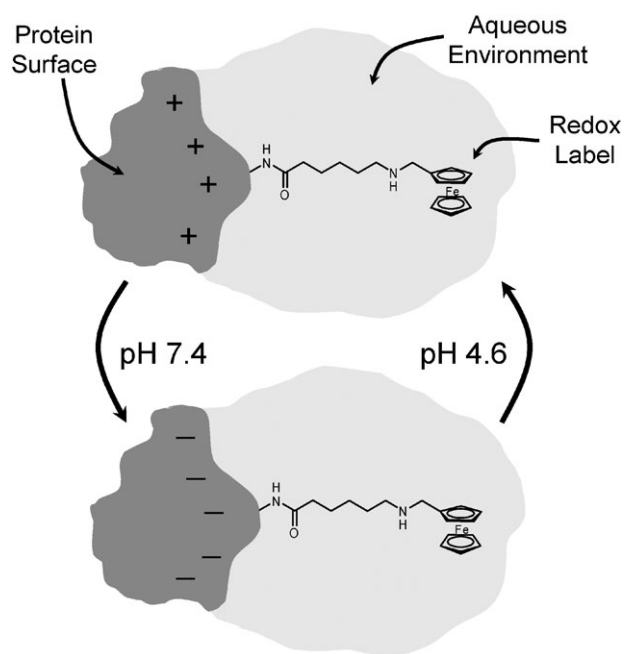


**Fig. 1** Cyclic voltammograms corresponding to ferrocene-labeled streptavidin bioconjugated on a biotinylated Au electrode in 0.1 M PBS (solid line) and 0.1 M NaAc/HAc (dashed line) buffer solutions. Scan rate: 50 mV s<sup>-1</sup>. Temperature: 298 K.

attribute these significant changes to the influence of the electrostatic environment introduced by the charged groups of the protein. To corroborate this assumption we measured the redox potential of the ferrocene linker, *i.e.* *N*-(ferrocenylmethyl)-6-amino hexanoic acid, dissolved in 0.1 M PBS buffer solution obtaining the value 0.365 V (*vs.* Ag/AgCl) (not shown), which is in close agreement with that reported by Padeste *et al.* under similar conditions.<sup>75</sup> As depicted in Fig. 1, the redox potential of the same electroactive center conjugated to the protein in a similar electrolyte environment is 0.29 V, thus giving a clear indication that the protein environment is the main factor responsible for the potential shift.

The direction of these changes can be described qualitatively by considering the relative stabilization of the different species involved in the redox couple. Firstly, we will analyze the differences coming into light when comparing the redox potential of the ferrocene linker in solution and confined to the conjugate layer in 0.1 M PBS buffer solution. Streptavidin (SAv) is a protein with an isoelectric point of 6 that is negatively charged at pH 7.4.<sup>76,77</sup> The ferrocene labels are in close proximity to the protein given by their 1–1.5 nm long linkers and consequently the electrostatic state of the protein will affect the stability of the redox species (Fig. 2).

The redox reaction of the ferrocene labels in solution involves the generation of ferricinium species in the charged environment provided by the electrolyte solution. On the other hand, in the redox reaction occurring at the bioconjugate the ferricinium species are surrounded not only by the electrolyte but also by the fixed charges of the protein that are also contributing to the electrostatic environment. Hence, the negative charge on the SAv protein contributes to further stabilizing the generation of positively charged ferricinium species.<sup>78</sup> In energy terms, this stabilization is observed as a potential shift of ~75 mV in the cathodic direction. This explains the differences of the formal potentials occurring when the electroactive labels are confined to the SAv bioconjugate layer at pH 7.4.



**Fig. 2** Simplified scheme describing the changes occurring in the surroundings of the redox labels upon changing the pH of the electrolyte.

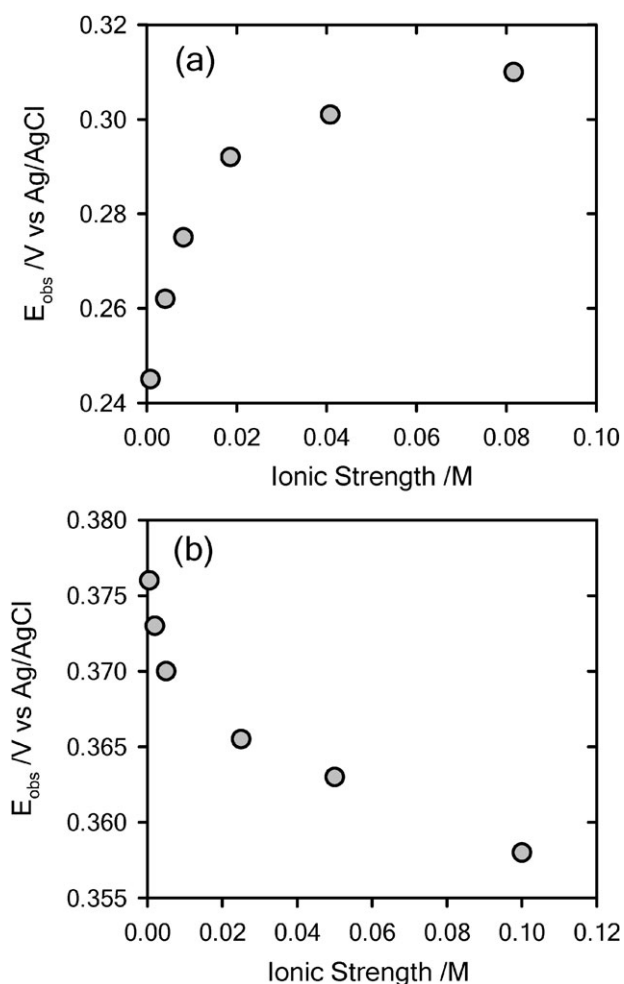
Next, we will rationalize the changes that occur in the redox-labeled bioconjugate if the pH is modified from 7.4 to 4.6, and hence the protein is positively charged. Fig. 1 shows that in 0.1 M NaAc/HAc buffer solution (at pH 4.6) the formal potential is shifted to more anodic values,  $E = 0.37$  V (*vs.* Ag/AgCl). This can be interpreted as the system requiring more energy to electrogenerate the cationic ferricinium species into a positively charged surrounding, with the long-range Coulombic interactions being predominantly repulsive.

This is a qualitative picture providing a descriptive view of the changes occurring in the formal potentials of the ferrocene labels when working below and above the isoelectric point of the protein. Moreover, it is also observed that the ionic strength of the buffer has a significant effect on the magnitude of the formal potential. This effect is even more pronounced at low ionic strength. At pH 7.4 a decrease in the ionic strength is reflected as a potential shift in the cathodic direction (Fig. 3a). Conversely, when decreasing the ionic strength at pH 4.6, the formal potential moves towards more anodic potentials (Fig. 3b).

This implies that the observed formal potential of the ferrocene labels is resulting from the interplay between the charges in the protein and the Coulombic screening of the ionic environment. It is worth noticing that these shifts in redox potentials are thermodynamic in nature given that both anodic and cathodic potentials shift together.<sup>73</sup>

To further analyze this situation, where the charges in the protein and the Coulombic screening of the species in solution play a major role on determining the redox potential of the electroactive labels, we will refer to the Gibbs free energy of the redox process ( $\Delta G_{\text{redox}}$ ) occurring at the electroactive label,





**Fig. 3** Dependence of the observed redox potential on ionic strength corresponding to: (a) PBS buffer solution (pH 7.4) and (b) NaAc/HAc buffer solution (pH 4.6). The electroactive surface was constituted by the Fe-SAv bioconjugated onto a biotinylated Au electrode.

As a starting point, we will describe the factors proposed by Moore *et al.*<sup>62</sup> which are responsible for determining the redox potential of electron transfer proteins. Obviously there are significant differences between an electron transfer protein and a redox-labeled protein. The former one is an *intrinsically* electroactive protein where the redox center is located at a very specific site into the protein environment. The other one is an *extrinsically* electroactive protein obtained by chemically coupling redox centers to lysine residues located at peripheral site of the protein. However, they have two aspects in common: (a) both of them refer to biomacromolecular entities having electroactive sites; and (b) the redox centers are in intimate contact with the protein environment.

That is the reason why we consider, and propose for the first time, the scenario introduced by Moore *et al.*<sup>62</sup> as valid for the initial description of  $\Delta G_{\text{redox}}$  in redox-active supramolecular bioconjugates.

In principle, the magnitude of  $\Delta G_{\text{redox}}$  should be ascribed to three major terms,

$$\Delta G_{\text{redox}} = \Delta G_{\text{cen}} + \Delta G_{\text{el}} + \Delta G_{\text{conf}} \quad (2)$$

where  $\Delta G_{\text{cen}}$  is the free energy difference resulting from bonding interactions at the redox center,  $\Delta G_{\text{el}}$  is the Gibbs energy difference resulting from electrostatic interactions between the redox center and the polar/charged groups within both the protein and the solvent, and  $\Delta G_{\text{conf}}$  is the Gibbs energy due to redox-state conformational differences, but these are considered negligible for simple electron transfer proteins.

It should be mentioned that  $\Delta G_{\text{cen}}$  is a major contributing term when comparing different electron transfer proteins where the same redox center possesses different coordination characteristics depending on the protein. In the present case, our analysis is based on studying the changes in the redox potential derived from variations in pH or ionic strength. So, this term plays no major role due to the nature of the linker of the redox labels remaining the same upon changes in ionic environment. As a result,

$$\Delta G_{\text{redox}} \sim \Delta G_{\text{el}} \quad (3)$$

There are also a number of factors contributing to  $\Delta G_{\text{el}}$ . This can be written as

$$\Delta G_{\text{el}} = \Delta G_{\text{ion}} + \Delta G_{\text{H}_2\text{O}} + \Delta G_{\text{int}} + \Delta G_{\text{surf}} \quad (4)$$

$\Delta G_{\text{ion}}$  describes the effect of the ionic environment in solution upon the redox energy. It mostly includes contributions from the non-specific *Debye-Hückel screening effect*, and in some cases, from specific ion-protein association.  $\Delta G_{\text{H}_2\text{O}}$  reflects the energy difference between the charge in the protein and the charge in the solvent. In accordance to Kassner<sup>79</sup> this factor is relevant when dealing with redox centers buried in the protein. In our case the redox centers are located at the periphery of the protein, being exposed to the solvent, which suggests that this term is not significant in our system.  $\Delta G_{\text{int}}$  is the Gibbs energy of the interaction between the redox center charge and other charges that are not exposed to the solvent. It has been suggested that this term is a significant contribution when the redox center is confined in the inner environment of the protein, where both charges are in a low dielectric medium.<sup>62</sup> In our case, the redox labels are not situated in the protein interior and, on the contrary, they are openly exposed to a high dielectric medium. This is the reason why we considered neglecting this term from the overall contributing factors.

Finally,  $\Delta G_{\text{surf}}$  is the Gibbs energy of interaction between the redox center charge and the protein charges exposed to the solvent. Regarding the latter, Rees suggested that this term plays a determinant role in influencing the magnitude of the redox potential in electron transfer protein.<sup>80</sup> Considering all the contributing terms, we can describe  $\Delta G_{\text{redox}}$  as

$$\Delta G_{\text{redox}} \sim \Delta G_{\text{ion}} + \Delta G_{\text{surf}} \quad (5)$$

Obviously these contributions will affect the thermodynamic state of the redox labels, which could be described as,

$$\Delta G_{\text{redox}} = \Delta G^\circ + RT \ln \frac{a_{\text{red}}}{a_{\text{ox}}} \quad (6)$$

where  $\Delta G^\circ$  is the Gibbs free energy under standard conditions and  $a_{\text{red}}$  and  $a_{\text{ox}}$  are the activities for the oxidized and reduced

species, respectively. Considering that  $\Delta G = -nFE$  we can turn eqn (6) into the Nernst equation,

$$E = E^\circ + \frac{RT}{nF} \ln \frac{a_{\text{ox}}}{a_{\text{red}}} \quad (7)$$

where  $E$  is the equilibrium potential,  $E^\circ$  is the standard electrode potential,  $R$  is the universal gas constant,  $F$  is the Faraday constant and  $n$  is the number of electrons involved in the electrochemical process.

As is well-known activities are parameters that reflect the thermodynamic state of the species in the system. The activity is given by

$$a_i = c_i \gamma_i \quad (8)$$

where  $c_i$  and  $\gamma_i$  are the concentration and the activity coefficient of species  $i$ , respectively.

The potential then becomes,

$$E = E^\circ + \frac{RT}{nF} \ln \frac{c_{\text{ox}}}{c_{\text{red}}} + \frac{RT}{nF} \ln \frac{\gamma_{\text{ox}}}{\gamma_{\text{red}}} \quad (9)$$

In most of cases, the activity coefficients are simply described as parameters indicating deviations from ideality. Notwithstanding this description, in our scenario they also account for the electrical contributions to the chemical potential of the electroactive labels. A theoretical estimation of the activity coefficient can be done using the Debye–Hückel theory where the activity coefficient is expressed as

$$\ln \gamma_i = - \frac{z_i^2 A \sqrt{I}}{1 + Ba_1 \sqrt{I}} \quad (10)$$

The parameter  $A$  depends on the relative permittivity and temperature. Typically, it has a value of  $0.509 (\text{L mol}^{-1})^{\frac{1}{2}} \text{cm}^{-1}$  for aqueous solutions at room temperature (298 K).  $z_i$  is the charge of species  $i$ .  $B$  is a constant for a specific temperature and has a value of  $0.329 \times 10^8 (\text{L mol}^{-1})^{\frac{1}{2}} \text{cm}^{-1}$  at 298 K. The parameter  $a_1$  corresponds to the effective radius of the ion which refers to the mean distance of closest approach.

Introducing eqn (10) into eqn (9) we obtain

$$E = E^\circ + \frac{RT}{nF} \ln \frac{c_{\text{ox}}}{c_{\text{red}}} - \frac{RTA(z_{\text{ox}}^2 - z_{\text{red}}^2)\sqrt{I}}{nF(1 + Ba_1\sqrt{I})} \quad (11)$$

Expression (11) describes the variation on the electrode potential upon variations in the ionic strength. This expression provides a more quantitative account on the effect of the ionic strength on the magnitude of the electrode potential. For the sake of clarity we will: (a) rename the electrode potential as “observed potential”,  $E_{\text{obs}}$ , (b) group and rename the first two terms at the right-hand side of eqn (11) as  $E^{\circ'}$ , and (c) introduce the values of the constants. Then,

$$E_{\text{obs}} = E^{\circ'} - 0.03 \frac{(z_{\text{ox}}^2 - z_{\text{red}}^2)\sqrt{I}}{1 + 0.329a_1\sqrt{I}} \quad (12)$$

or

$$E_{\text{obs}} = E^{\circ'} - 0.03 (z_{\text{ox}}^2 - z_{\text{red}}^2)f(I) \quad (13)$$

where

$$f(I) = \frac{\sqrt{I}}{1 + 0.328a_1\sqrt{I}} \quad (14)$$

In the case of redox centers confined in biomacromolecular environments, a more accurate description of  $z_{\text{ox}}$  and  $z_{\text{red}}$  is given by the net charges of the electroactive biomolecules in the reduced and oxidized states ( $q_{\text{red}}$  and  $q_{\text{ox}}$ ), thus giving

$$E_{\text{obs}} = E^{\circ'} - 0.03 (q_{\text{ox}}^2 - q_{\text{red}}^2)f(I) \quad (15)$$

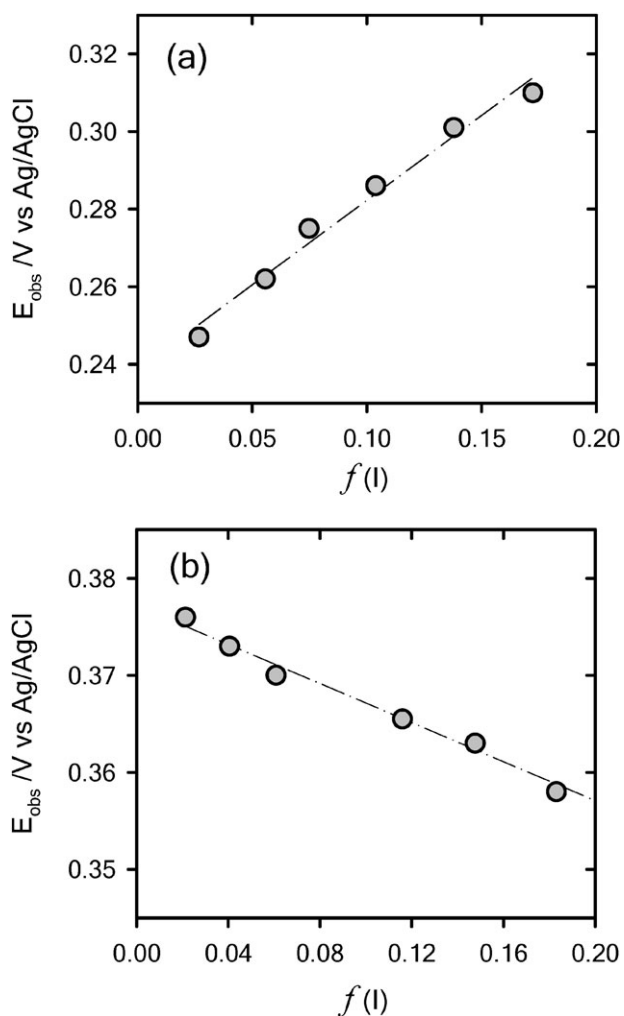
The replacement of  $z_{\text{ox}}$  and  $z_{\text{red}}$  by  $q_{\text{red}}$  and  $q_{\text{ox}}$  was formerly introduced by Margalit and Schejter.<sup>67</sup> Later on, the validity of this approach was corroborated using an elegant set of experiments with modified proteins. Aviram *et al.* partially modified cytochrome *c* by progressive maleylation of lysine groups, hence introducing carboxylate groups into the protein in a controlled manner.<sup>81</sup> This enabled them to obtain a set of cytochrome *c*'s with tailored net charge. The fitting of their results using eqn (15) showed very good agreement with the expected (actual) net charge of the protein, demonstrating that the expression of Margalit and Schejter provided realistic information on the electrostatic environment of the redox center. Notably, this also indicates that even if the Debye–Hückel theory is only applicable in low ionic strength ranges it could be used as a primary tool to obtain a quite good description of the electrostatic interactions in the surroundings of the redox centers. Very recently, Petrović *et al.* used the same approach to elucidate the role of the ionic strength on the formal potential of cytochrome *c* immobilized on self-assembled monolayers.<sup>82</sup> They found that the apparent charge of the protein was strongly influenced by the nature of the anchoring layer, thus obtaining positive or negative slopes for cyt *c* immobilized on COOH- or pyridyl-terminated SAMs, respectively.

In this scenario the slopes of  $E_{\text{obs}}$  vs.  $f(I)$  provide information on the net charges of the protein environment where the redox center is incorporated. In order to generate the function  $f(I)$  we used the molecular size of the ferrocene labels as an estimation of the parameter  $A$ , 0.7 nm.<sup>83</sup> The representation of  $E_{\text{obs}}$  as a function of  $f(I)$  at pH 7.4, describes a linear correlation with a positive slope corresponding to 0.43 (Fig. 4a).

Considering that  $q_{\text{ox}} = q_{\text{red}} + 1$  this implies that at pH 7.4

$$(q_{\text{ox}}^2 - q_{\text{red}}^2) = 2q_{\text{red}} + 1 = -14.3, \quad (16)$$

thus giving an indication that at pH 7.4 the effective charge of the protein affecting the molecular-level environment of the redox label is *ca*  $-7.6$ . This value differs from estimations of surface charge reported by Leckband and co-workers who estimated  $-2$  at pH 7.6.<sup>84</sup> The reasons to explain these differences could be attributed to the fact that we are working with a modified SAV. The effective charge of the protein implies a delicate balance between its charged groups. This balance can be affected by introducing modifications into the residues. For example, the maleylation of five lysine groups in cytochrome *c* impacts on the generation of ten negative charges. To introduce the ferrocene labels in the SAV requires modifying lysine residues, which naturally affects the balance



**Fig. 4** Representation of  $E_{\text{obs}}$  as a function of  $f(I)$  (cf. eqn (15)) obtained for the ferrocene-labeled bioconjugate in: (a) PBS buffer solutions (pH 7.4) and (b) NaAc/HAc buffer solutions (pH 4.6) of different ionic strengths.

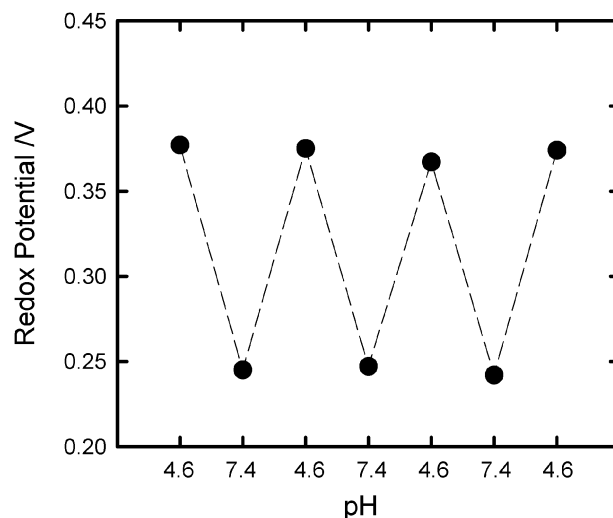
of charged residues in the protein explaining the rather high value of effective negative charges on the protein.

On the other hand, the dependence of  $E$  on  $f(I)$  at pH 4.6 also describes a linear correlation (Fig. 4b), but with a negative slope corresponding to  $-0.1$ . Using the same procedure as described above we obtained an effective charge for the ferrocene-modified protein in the reduced form equal to 1.2. Once again, we need to note that this value reflects the effective charge in the surrounding of the redox label and may differ from that corresponding to the surface charge of the unmodified SAV.<sup>84</sup> The reason for this difference lies in the fact that the surface lysine groups that are involved in the balance of charges of the protein structure were modified by the conjugation of the label. This observation indicates that the extent of labeling is another variable that could affect the ionic strength and pH dependency of the redox potential. Finally, considering the similarities between redox proteins (*intrinsically electroactive* proteins) and redox-labeled proteins (*extrinsically electroactive* proteins) we assume that specific binding of ions (at low ionic strength conditions) could also affect the magnitude of the redox potential. This assumption is based on

experimental work reported by Gopal *et al.*<sup>85</sup> using horse, bovine and tune cytochrome *c* in which the redox potential was influenced by the ion binding of different species like phosphate, chloride or tris-cacodylate.

As shown, the framework previously developed to conceptualize the formal potential of *intrinsically* electroactive proteins, like cytochrome *c*, has demonstrated to be a valuable tool to gain understanding on the molecular level characteristics and the electrochemical behaviour of redox-active supramolecular assemblies, in which the electroactive centers are chemically bound to the protein architecture.

These results quantify how sensitive the redox centers are to the characteristics of the local environment. This framework is the platform for controlling and tuning the redox energy of the electroactive centers in the supramolecular bioconjugate in a predictable manner. For example, we already discussed that at low ionic strengths the Coulombic screening of the charged environment is weak and the redox centers “feel” with more intensity the nature of the charges at the protein surface. This implies that depending on the nature and magnitude of these charges we can significantly shift the redox potential in opposite directions by simply changing the pH at low ionic strengths. This can be used as a tool for tuning the redox potential of the electroactive interfacial architecture over a wide range of potentials using the same supramolecular bioconjugate. Fig. 5 displays the reversible changes in redox potential upon variations in pH from 4.6 to 7.4 at low ionic strengths. The tuning of the redox energy of the ferrocene centers is simply achieved by modifying the local environment provided the charged streptavidin matrix. In this case, pH changes from 4.6 to 7.4 (at low ionic strengths) lead to a potential shift of  $\sim 130$  mV. This represents a very interesting case of a pH-tunable electrochemical biointerface entirely based on supramolecular bioconjugates, where the bio-macromolecule is the vehicle for *externally* tuning the redox



**Fig. 5** Reversible changes observed in the redox potential of the surface-confined ferrocene-labeled streptavidin bioconjugate upon changes in pH. The value of the redox potential was measured *versus* a Ag/AgCl reference electrode. The ionic strengths of PBS (pH 7.4) and NaAc/HAc (pH 4.6) buffers were  $8 \times 10^{-4}$  M and  $5 \times 10^{-4}$  M, respectively.

state of the electroactive centers. It is worth mentioning that pH-tunable electrochemical interfaces are commonly encountered in hydroquinone-related organic films.<sup>86</sup> However, this is the first example of a pH-responsive electrochemical interface based exclusively on man-made bio-inorganic assemblies. This an example illustrating the richness of bio-inorganic hybrids to act as building blocks for designing functional materials in a predictable manner.

## Conclusions

In summary, in this work we have studied the influence of electrostatic microenvironment on the redox potential of ferrocene-labeled supramolecular bioconjugates. Our findings indicate that the charge of the protein conjugate plays a determinant role in stabilizing the species involved in the redox reaction. Depending on the pH, the sign of the fixed charges in the protein layer can be modified, thus impacting on the magnitude of the redox potential. The formation of cationic ferricinium species is stabilized at pH 7.4, where the protein bioconjugate is negatively charged. This is experimentally observed as a cathodic shift of the formal potential respect to the redox probe in solution. Conversely, at pH 4.6 the positive fixed charge in the protein layer leads to a scenario where the ferricinium species are electrogenerated in a surrounding dominated by repulsive Coulombic interactions. The ionic strength plays a central role in screening the stabilizing or repulsive interactions. At pH 7.4, increasing the ionic strength is reflected as a decrease in the stabilizing effect of the fixed negative charges. This is a consequence of a more efficient screening of the stabilizing interactions between the ferricinium ions and the negative fixed charges in the surroundings. This provokes an anodic shift in the redox potential upon increasing the buffer concentration.

On the contrary, the increase on the ionic strength at pH 4.6 decreases the repulsive Coulombic interactions where the ferricinium is electrogenerated. As a consequence, increasing the buffer concentration is reflected as a cathodic shift in  $E_{\text{obs}}$ .

The dependence of the redox potential on pH and ionic strength can be interpreted in a more quantitative manner by introducing an approach similar to that formerly described by Margalit and Schejter<sup>67</sup> to rationalize the electrochemical behaviour of cytochrome *c*.

To the best of our knowledge, this approach has been neither extended nor corroborated in case of redox-labeled supramolecular bioconjugates (*extrinsically* electroactive proteins). We consider that this approach would introduce new tools, directions and alternatives to the study and rational design of redox-active bioconjugates. It enables a more detailed description on how the charged groups of the bioconjugate affect the electrostatic (and thermodynamic) state of the redox labels, also accounting for the screening of the long range electrostatic interactions between the redox centers and the protein charged groups surrounding them. Moreover, it also provides a framework to predict formal potential variations in redox-active biomolecular assemblies and charged supramolecular architectures. This can be used as a valuable tool to design redox-active functional assemblies with tailorable structure–function properties. The potential of the

approach was illustrated by the creation of a pH-tunable bioelectrochemical interface using redox-active supramolecular conjugates as building blocks.

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