Amplified vibrational circular dichroism
Rosa Domingos, S.M.

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LOCAL VCD ENHANCEMENT IN SWITCHABLE FERROCENE-LINKED PEPTIDES

We investigate the space dependence of VCD signal-amplification due to low-lying electronically excited states by VCD studies on synthetic ferrocene-linked peptides. Electrochemical switching of the redox state of ferrocene covalently bound to a peptide-like bio-functionality enables us to induce an amplification of the VCD signal intensity of vibrational modes along the peptide backbone. Investigation of the distance dependence of the amplification indicates a localized character of the enhancement. These results demonstrate that incorporation of a redox-switchable unit like ferrocene provides a powerful means to probe locally — and at user-defined locations — the structure of large systems.
8.1 INTRODUCTION

The functionality of biomolecular systems is in general related to a spatially restricted region of the molecule such as, for example, the active site of an enzyme. For a detailed understanding of the relation between structure and functionality of these systems one ideally would like to be able to zoom in to that specific part of the system with a technique that is able to characterize its electronic and spatial structure, and observe changes that occur upon a change of the active site, for example, as the result of substrate binding. Also, in many cases a local change, either induced by external stimuli or by binding, results in global changes of the system. In a similar vein one would like in these cases as well to be able to probe specific locations without interference of the rest of the system. Ideally, one would like to have the possibility to place a molecular beacon at any specific location in a molecular system so as to "illuminate" only that specific environment.

VCD is intrinsically capable of probing the spatial structure and conformational changes of site-specific moieties in large molecules. However, since all parts of the molecule contribute to the VCD spectrum, band overlap generally results in a congested spectrum with limited information regarding specific oscillators. Inspired by the enhancements observed and discussed in the previous chapters, we therefore propose here the implementation of a VCD amplifier, which (a) can be covalently bound to a specific location within a molecule and (b) can be switched chemically to provide the required environment for VCD enhancement in a spatially-restricted part of the target molecule (Fig. 39). In this way, one would be able to spectrally isolate the target region of the molecule by means of an amplified VCD response of the oscillators in the near vicinity of the amplifier. The studies reported in Chapter 7 demonstrate that the observed VCD enhancements are to a certain extent localized. Here, we investigate the space-dependence of the amplified VCD response in a systematic way using an electroactive moiety, which is covalently coupled to a chosen location in a series of peptides. With this approach we are able to switch the amplifying source ON and OFF simply by adjusting the electrochemical potential, and locally amplify the
VCD response. By subtracting the VCD spectra in the ON and OFF states, we will show that it is possible to isolate the VCD response of the local environment from the total VCD spectrum. We believe that this approach might ultimately lead to the development of a chiral-sensitive probe with which one can investigate local effects such as drug-receptor interactions,[166] biosensing key-lock mechanisms[167, 168, 169] and active sites in proteins and enzymes.[170]

Figure 39: Crystal structure of catalytic antibody Fab 1345 (PDB 1A3L), which contains a ferrocene moiety in its active site [171].

As a first step in this direction, we use the VCD-OTTLE setup[75] to study the VCD response of a series of peptides covalently bound to a switchable amplifier and investigate the amplification effect when the electronic structure of the electroactive group is altered. For this specific purpose, we have chosen ferrocene (Fc) as the external VCD amplifier. In its neutral Fe\(^{2+}\) form, ferrocene has a closed-shell electronic configuration with electronically excited states well separated from the electronic ground state. In contrast, the one-electron oxidized ferricenium cation exhibits an open-shell configuration (Fe\(^{3+}\)) with low-lying electronically excited states that are responsible for enhanced VCD. The
electroactive group can be electrochemically switched ON (open-shell) and OFF (closed-shell) thus providing a handle for controlled manipulation of the magnitude of the VCD response. An understanding of the spatial sensitivity of the VCD amplification effect is required in order to predict which molecular entities will be influenced most upon activation of the redox-switch. We thus investigate the distance-dependence of the VCD amplification in backbone amide I’ vibrational modes of ferrocenyl-based peptides using VCD-spectroelectrochemistry.\[75]\ The relative ease with which this unit can be incorporated at specific locations within a larger molecular system holds great promise for its application to probe local structure using VCD.

### 8.2 Experimental Methods

#### 8.2.1 Synthesis and Characterization

Three prototypes were synthesized for this study: an enantiomeric pair (L/D) of Fc-based alanine methyl-ester dipeptides and a L-stereoisomer of a Fc-based tripeptide with amino acid sequence: Ala-Pro-Ala. Fig. 40 and 41 display the chemical structures of the L-stereoisomers of the dipeptide and tripeptide Fc-prototypes, respectively.

![Chemical structure of N-(ferrocenylcarbonyl)-L-alanyl-L-alanine methyl ester. Abbreviation: Fc-(L)-Ala-Ala.](image)

**Figure 40:** Chemical structure of N-(ferrocenylcarbonyl)-L-alanyl-L-alanine methyl ester. Abbreviation: Fc-(L)-Ala-Ala.

#### 8.2.1.1 General procedure for the coupling of ferrocene to the peptides

To a solution of ferrocene carboxylic acid (1:1 equivalent; 100 – 150 mg) in acetonitrile (5 ml) 3 equivs of DIPEA and 1.5 equiv of HCTU were
added. The resulting mixture was stirred for 30 min, after which a solution of the peptide ester (100–150 mg) in acetonitrile (5 ml) was added. After checking the conversion, the reaction mixture was concentrated in vacuo, and the residue was dissolved in EtOAc (50 ml) and Et₂O (50 ml), followed by addition of a few milliliters of CH₂Cl₂. This mixture was washed twice with 5% aqueous bicarbonate (20 ml), once with water (20 ml), twice with 17% aqueous NH₄Cl, six times with water (20 ml) and once with brine (20 ml), then dried (Na₂SO₄) and concentrated in vacuo. The residue was purified by flash chromatography (SiO₂, CH₂Cl₂/MeOH – 98/2 v/v). Yields ranged from 30 to 67%.

8.2.1.2 Characterization

1. N-(ferrocenylcarbonyl)-L-alanyl-L-alanine methyl ester. ¹H NMR (400 MHz, CDCl₃) δ 6.65 (d, J = 6.5 Hz, 1H), 6.23 (d, J = 7.6 Hz, 1H), 4.72 (s, 1H), 4.66 (s, 2H), 4.65 – 4.55 (m, 2 H), 4.37 (s, 2H), 4.21 (s, 5H), 3.77 (s, 3H), 1.47 (d, J = 7.0 Hz, 3H), 1.45 (d, J = 7.2 Hz, 3H); TLC : Rf ≈ 0.4 (CH₂Cl₂/MeOH – 95/5).

2. N-(ferrocenylcarbonyl)-D-alanyl-D-alanine methyl ester. ¹H NMR (400 MHz, CDCl₃) δ 6.75 (d, J = 6.9 Hz, 1H), 6.28 (d, J = 7.4 Hz, 1H), 4.72 – 4.65 (m, 3H), 4.58 (p, J = 7.2 Hz, 1H), 4.36 (s, 2H), 4.21 (s, 3H), 3.77 (s, 3H), 1.47 (d, J = 6.8 Hz, 3H), 1.45 (d, J = 7.2 Hz, 3H); TLC : Rf ≈ 0.5 (CH₂Cl₂/MeOH – 95/5).

3. N-(ferrocenylcarbonyl)-L-alanyl-L-prolinyl-L-alanine methyl ester. ¹H NMR (400 MHz, CDCl₃) δ 7.02 (d, J = 7.1 Hz, 1H), 6.59 (d, J = 7.6 Hz, 1H), 4.90 (p, J = 6.8 Hz, 1H), 4.70 – 4.68 (m, 2H),

Figure 41: Chemical structure of N-(ferrocenylcarbonyl)-L-alanyl-L-prolinyl-L-alanine methyl ester. Abbreviation: Fc-(L)-Ala-Pro-Ala.
4.62 – 4.58 (m, 1H), 4.55 – 4.50 (m, 1H), 4.34 (m, 2H), 4.21 (m, 5H), 3.79 – 3.72 (m, 1H), 3.75 (s, 3H), 3.66 – 3.61 (m, 1H), 2.32 – 2.28 (m, 1H), 2.16 – 1.98 (m, 3H), 1.46 (d, J = 6.8 Hz, 3H), 1.40 (d, J = 7.2 Hz, 3H); TLC : Rf ≈ 0.3 (CH₂Cl₂/MeOH – 95/5).

8.2.2 Spectroelectrochemical VCD measurements

All VCD spectra displayed in this Chapter have been measured using the VCD–OTTLE[75] cell. For experimental details see Chapter 3 (Section 3.2). Working with ferrocene as the molecular entity that can be electrochemically switched between redox states (Fe²⁺/Fe³⁺), allows us to use the normal short-path OTTLE[39] configuration but keeping the circular aperture in the working electrode according to the design of the VCD-OTTLE[75] cell. In this manner, we decrease the optical pathlength and increase the concentration of the sample solutions. Due to the performance of the short-path OTTLE cell we can oxidize quickly and keep the product stable for many averaging scans to improve the signal-to-noise ratios. In Fig. 42 we show thin-layer cyclic voltammograms of the Fc-(L)-Ala-Ala and Fc-(L)-Ala-Pro-Ala compounds. In both cases we observe full chemical reversibility (despite the irregular shapes of anionic waves and cathodic counter-waves) i.e., stability of both redox states.

![Cyclic voltammograms](image)

Figure 42: Thin-layer cyclic voltammograms (ν = 5 mVs⁻¹) of Fc-(L)-Ala-Ala (right) and Fc-(L)-Ala-Pro-Ala (left) obtained using the VCD-OTTLE cell. Both solutions were prepared in dry CD₃CN (10⁻¹ M Bu₄NPF₆).
8.3 Theoretical Methods

Density Functional Theory (DFT) calculations were carried out with Gaussian 09\[^{23}\]. Ground-state geometry optimizations and harmonic vibrational frequencies were computed using the B3LYP hybrid functional, which includes the Becke three-parameter exchange\[^{105}\] and the Lee, Yang and Parr correlation functionals\[^{107}\]. The LANL2DZ effective core potential (ECP) was used for the Fe core element, whereas the 6-31+G(d) basis set was used for all other elements. Solvent effects were simulated with a polarizable continuum model (PCM). Excitation energies and electronic magnetic transition-dipole moments were calculated using time-dependent DFT.

8.4 Results

In the following, the designations OFF and ON will be utilized to describe the two relevant electronic configurations of the electroactive group (Fc). We will thus refer to ferrocene in its neutral closed-shell configuration (Fe\(^{2+}\), singlet, no unpaired electrons) as the OFF configuration, whereas the oxidized, cationic open-shell configuration (Fe\(^{3+}\), doublet, one unpaired electron) will be referred to as the ON configuration. The ON configuration generates an electronic manifold with low-energy electronically excited states, i.e., it "activates" the VCD amplification, whereas the OFF configuration turns off the amplification by returning the electronic manifold to its original configuration, without low-lying electronically excited states.

8.5 FC-ALA-ALA

In Fig. 43 we show the molecular structure of the first prototype, a ferrocene moiety connected to L-Ala-L-Ala-methyl-ester (Fc-(L)- Ala-Ala). The chemical groups that serve as a probe for the spatial extension of VCD enhancement are labeled based on their increasing distance from
the electroactive group and referenced by the ruler plotted next to the molecular structure as a guide to the eye.

![Optimized molecular structure of Fc-(L)-Ala-Ala obtained at the DFT level of theory. A schematic ruler is plotted next to the peptide backbone to highlight the distance between the ferrocene moiety, and the amide and ester groups. The numbers correspond to the associated vibrational modes of amide and ester groups assigned in Fig. 44.](image)

Fig. 44A shows infrared absorption spectra of Fc-(L)-Ala-Ala in the OFF (solid line) and ON (dashed line) configurations. We can promptly assign three vibrational modes, which are labeled in Fig. 44 according to their position as displayed in Fig. 43. In the OFF configuration, there are two amide I’ vibrational modes (1 and 2) at 1650 and 1684 cm⁻¹, respectively. Band 3 at higher frequency (∼1750 cm⁻¹) is assigned to the methyl ester C=O-stretching. Upon electrochemical switching to the ON configuration, amide I’ mode 1 is shifted to the blue by 19 cm⁻¹, while amide I’ mode 2 is blue shifted by only 3.5 cm⁻¹. The C=O methyl ester vibrational mode (3) does not undergo any frequency shift (see Table 7). Our spectral window shows well separated bands for the vibrational modes under investigation, thus allowing for a clear comparison of VCD intensities (Fig. 44).

Fig. 44B displays the VCD spectra for the OFF/ON configurations. Comparison of the VCD signal intensities for vibrational modes 1, 2 and 3 (OFF) and corresponding 1’, 2’ and 3’ (ON) readily indicates an am-
Figure 44: Infrared absorption (A) and VCD spectra (B) of 10^{-2} M Fc-(L/D)-Ala-Ala-Ester in CD_{3}CN (10^{-1} M Bu_{4}NPF_{6}, 200 \mu m optical path-length). The VCD spectra of the ON configuration are offset for clarity. Panel C displays the difference spectrum of the VCD spectra in the ON and OFF configurations.

amplified VCD response in the ON configuration. As a direct measure of the amplification magnitudes we use the anisotropy factors \( g = \Delta \varepsilon / \varepsilon \) of each vibrational mode. The results reported in Table 7 indicate that the signal intensity of the amide I' (Ala_{1}) vibration is amplified most, while amide I' (Ala_{2}) is only slightly enhanced. The C=O methyl ester (Ala_{3}) VCD intensity lies within the noise of the measurement and therefore can not be evaluated accurately. The amplification thus seems to be stronger for the amide I' mode closest to the ferrocene moiety as compared to the other amide I' and C=O methyl-ester modes. In Fig 44C the difference spectrum ON-OFF is depicted. Since the intensities of bands
in the VCD spectrum of the system in the ON configuration are strongly dependent on the distance from the amplifying unit, this spectrum is in fact zooming in on that part of the system that is within amplifying range. As a result, signals from the ester moiety are suppressed, while the amide I’ oscillators in position 1 and 2 are highlighted.

Table 7: Frequency shifts and anisotropy factors \(g = \Delta \epsilon / \epsilon\) for the vibrational modes 1, 2 and 3 in the OFF and ON configurations of Fc-(L)-Ala-Ala.

<table>
<thead>
<tr>
<th>Modes</th>
<th>OFF</th>
<th>ON</th>
</tr>
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<tbody>
<tr>
<td>(\text{Amide I'}_1(\text{Ala}_1))</td>
<td>(19.0) 1024 0.01 9.7 \times 10^{-6}</td>
<td>1380 0.10 7.2 \times 10^{-5}</td>
</tr>
<tr>
<td>(\text{Amide I'}_2(\text{Ala}_2))</td>
<td>3.5 820 0.02 2.4 \times 10^{-5}</td>
<td>840 0.05 5.9 \times 10^{-5}</td>
</tr>
<tr>
<td>Ester (\text{Ala}_3)</td>
<td>0 640 0.01 1.6 \times 10^{-5}</td>
<td>650 NA NA</td>
</tr>
</tbody>
</table>

8.6 FC-ALA-PRO-ALA

The above observations indicate that the oscillators close to the ferrocene are mostly subject to VCD enhancement. We extended the probing range to gain further insight in the distance dependence of the VCD enhancement. To that purpose, we compared the amplification factors of VCD signals in a tripeptide (Ala-Pro-Ala) attached to ferrocene. Fig. 45 shows the molecular structure of Fc-(L)-Ala-Pro-Ala (OFF) obtained after geometry optimization at the DFT level of theory. Ala-Pro-Ala was specifically chosen for this study because of its well separated amide I’ frequencies, allowing us to separate the VCD peaks for better evaluation of the signal intensity and amplification. In this particular case, the proline residue has a distinct red-shifted amide I’ vibrational frequency compared with that of Alanine. Moreover, the electron-withdrawing character of the amide functionality Ala1 leads to distinct amide I’ frequencies for the alanine moieties. Fig. 46A shows the infrared absorption spectrum of Fc-(L)-Ala-Pro-Ala (OFF configuration) which shows three amide I’ (Ala1, Pro2 and Ala3) and one methyl-ester C=O-stretching (Ala4) modes in the
spectral window. As expected, all three modes have distinct frequencies as evidenced by the Lorentzian curves that fit the FTIR spectrum. We assign the frequencies of the four modes as follows: Pro₂ (1642 cm⁻¹), Ala₁ (1657 cm⁻¹) Ala₃ (1687 cm⁻¹) and Ala₄ (1745 cm⁻¹). The numbers as subscripts corresponding to the numbered positions in Fig. 45. In Fig. 46B we show the VCD spectrum of Fc-(L)-Ala-Pro-Ala with ferrocene in the OFF configuration. Three features are readily identified in the spectrum and assigned according to the numbering in Figs. 46B and 45. The extinction coefficients (ε) and differential extinction coefficients (Δε) are listed in Table 8.

Figs. 46C – D show the infrared absorption and VCD spectra of Fc-(L)-Ala-Pro-Ala in the ON configuration. All infrared bands are blue-shifted except for Ala₄ (see Table 8), although the modes are not equally affected by the oxidation of the ferrocene moiety (Table 8). As expected, the overall VCD signal intensities (Fig. 46D) are considerably enhanced compared with those of the the bands in the OFF configuration. However, the amplification factors for the individual modes differ consid-
Figure 46: Infrared absorption (A,C) and VCD spectra (B,D) of $10^{-2}$ M Fc-(L)-Ala-Pro-Ala in CD$_3$CN ($10^{-1}$ M Bu$_4$NPF$_6$, 200 µm optical path-length), for the OFF and ON configurations, respectively. Panel E displays the difference spectrum of the VCD spectra in the ON and OFF configurations.

erably. In Table 8 we list the extinction coefficients ($\varepsilon$) and differential extinction coefficients ($\Delta\varepsilon$) for the vibrational modes of Fc-(L)-Ala-Pro-Ala. Again, in order to accurately compare the intensity enhancement for each mode, we determine their anisotropy factors ($g = \Delta\varepsilon/\varepsilon$) in the OFF and ON configurations. In Fig. 47 we plot the amplification factor, defined as the ratio $g'/g$, as a function of the distance of the oscillator to the electroactive moiety for Fc-(L)-Ala-Ala and Fc-(L)-Ala-Pro-Ala. The
correlation between \( g'/g \) and the distance of the amide groups to the Fc moiety provides clear evidence that the enhancement phenomenon decays strongly with increasing distance to the amplifier. Fig. 46E displays the ON-OFF difference VCD spectrum. In this case we observe that the signals from oscillators 3 and 4 are suppressed, and that the amplifying unit once again zooms in on oscillators 1 and 2.

![Graph showing VCD amplification factors as a function of distance to the switch](image)

Figure 47: VCD amplification factors \((g'/g)\) as a function of the distance (number of covalent bonds) from the electroactive group to each of the indicated functional groups of Fc-(L)-Ala-Ala and Fc-(L)-Ala-Pro-Ala (for numbering see Figs. 43 and 45).
8.7 LOW-LYING ELECTRONICALLY EXCITED STATES

The results presented above convincingly give evidence for the local character of the VCD enhancement in these peptides. In the following, we will analyze the amplification mechanism in terms of the effect of low-lying electronically excited states. For this purpose we use time-dependent DFT to evaluate the electronic-dependent terms in Eq. 43. In Chapter 2, we have shown how the intrinsic low intensity of VCD signals is strongly dependent on the weak contribution of the electronic component of the magnetic transition-dipole moment to the rotational strength (Eq. 16). Vibronic coupling is responsible for an increased mixing of BO states with the ground state, and leads to an enhancement of VCD signals. The electronic contribution to the magnetic component of the rotational strength is described by Eq. 43. We can thus evaluate the relative contributions of each electronic state to the VCD signal intensity, by determining their electronic excitation energy, \( W_K - W_G \), and associated magnetic transition-dipole moment from the electronic ground-state \( |\psi_G\rangle \).

8.7.1 Fc-Ala-Ala, Fc-Ala-Pro-Ala

In Figs. 48A and 48C we show the predicted excitation energies of the first ten electronically-excited states of Fc-(L)-Ala-Ala and Fc-(L)-Ala-Pro-Ala, respectively, for the OFF and ON configurations. Interestingly, we find that in the ON-configuration the two lowest electronic transitions are at much lower energies compared than in the OFF-configuration. It is thus reasonable to expect a strong contribution from those two electronic transitions to the observed enhanced VCD intensities. In Figs. 48B and 48D we plot the calculated electronic magnetic transition-dipole moment for each of the electronic excitations divided by their respective excitation energies. These ratios are simply the expansion coefficients in Eq. 43. Figs. 48B and 48D strongly suggest that the first electronically excited state has a dominating role in the VCD amplification. Moreover, evaluating the ratio of the coefficients in the OFF and ON configurations
Figure 48: Excitation energies (A,C) and amplification coefficients (B,D) for the first ten electronically excited states of Fc-(L)-Ala-Ala and Fc-(L)-Ala-Pro-Ala, respectively. The black squares represent the OFF configuration while the red circles represent the ON configuration.

for the first electronic excitation yields an amplification factor ON/OFF of 28.7. It is interesting to note that solely on account of the first electronic state one can expect amplification of the VCD intensity by more than one order of magnitude. This result is in agreement with previous conclusions indicating that in some cases the VCD magnitudes can be accurately estimated using a simple two-level system (see Chapter 6). Fig. 48 shows that the second electronic transition is also considerably lowered in excitation energy upon switching from the OFF to ON configuration. However, the magnetic transition dipole moment to this state is significantly smaller than the transition moment to the first state, and its contribution can thus be neglected.

Considering the electron-withdrawing property of amide groups, it is surprising to see that for both peptides the ON configuration relaxes to a geometry in which the angle between the amide plane and the aro-
matic rings of ferrocene is larger than for the OFF configuration (see Fig. 49 and Table. 9). This indicates a weaker electron-withdrawing effect in the ON configuration. Nevertheless, comparison of the angle in the relaxed geometry for Fc-(L)-Ala-Pro-Ala and Fc-(L)-Ala-Ala shows a much smaller angle for the latter. Although not substantial, this difference might explain the smaller amplification observed for the amide functionality in the Ala directly connected to Fc in Fc-(L)-Ala-Ala compared with that in Fc-(L)-Ala-Pro-Ala (see Fig. 47).

Table 9: Angles ($\phi$) between the Amide I' (C=O) plane and the ferrocene aromatic rings for the Ala-Ala and Ala-Pro-Ala peptides in the OFF and ON configuration.

<table>
<thead>
<tr>
<th></th>
<th>OFF</th>
<th>ON</th>
</tr>
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<tbody>
<tr>
<td>Ala-Ala</td>
<td>6.1°</td>
<td>24.6°</td>
</tr>
<tr>
<td>Ala-Pro-Ala</td>
<td>4.7°</td>
<td>11.1°</td>
</tr>
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</table>

Figure 49: Overlap of the OFF/ON (white/blue) calculated structures of Fc-Ala-Ala (top) and Fc-Ala-Pro-Ala (bottom). The angles between the amide functionality and the ferrocene aromatic ring are given in Table. 49.
8.8 Conclusions and Outlook

In the present chapter we have shown how amplified VCD can be used to zoom in into specific parts of biomolecular systems. To this purpose, we have investigated switchable ferrocenyl-based peptides, which are prototypical examples of how an amplifying unit (ferrocene) would be incorporated into a larger structure. In the OFF configuration no amplification occurs, in the ON configuration low-energy excited states are created that were found to enhance the VCD by more than an order of magnitude. The observed distance dependence of the VCD enhancement along the peptide backbone provides strong evidence that the amplification is a local phenomenon.

We have shown how subtraction of VCD spectra obtained under amplifying and non-amplifying conditions allows us to suppress signals from oscillators that are further removed from the amplifying unit. As a perspective for the future, Fig. 50 shows a schematic outlook for the application of locally amplified VCD for the investigation of site-specific chiral molecular targets. The highlighted region of the molecule contains the electroactive group (Fig. 50, top panel) which is embedded within the molecule. Subtracting the VCD spectra in the ON and OFF states (Fig. 50, lower panel) gives rise to signals that are associated with oscillators in the near vicinity of the electroactive group. The incorporated VCD amplification switch thus effectively allows us to turn VCD into a zero-background technique (subtraction of the ON and OFF VCD spectra leads to null signals for spatial regions not connected to the switch), and paves the way towards a unique way to spectrally resolve protein local structure both under static as well as dynamic conditions.
Figure 50: Schematic figure describing amplified VCD as a zero background technique using ON/OFF subtraction to zoom in into large biomolecular systems. This protein, catalytic antibody Fab 1345 (PDB 1A3L), contains a ferrocene moiety in its active site. [171].