Fluorescence Detected Circular Dichroism. Theory

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Abstract: The relation between fluorescence detected circular dichroism (FDCD) and the standard method of measurement of circular dichroism (CD) is obtained. The effects of concentration on the signal and the signal-to-noise ratio are described. As the concentration approaches zero the signal approaches the Kuhn asymmetry factor, $\Delta\epsilon_F/\epsilon_F$, and the signal-to-noise ratio approaches a maximum. Only transitions, which after absorption of light lead to fluorescence, contribute to the molar circular dichroism, $\Delta\epsilon_F$. The contributing chromophore may fluoresce directly or transfer energy to a fluorophore. Methods for interpreting the FDCD in terms of molecular structure are discussed. Comparison of FDCD and CD for flexible molecules should be particularly interesting.

The method of fluorescence detected circular dichroism has been presented[1,2] and applications have been made to transfer RNA.[3,4] Fluorescence detected magnetic circular dichroism has also been reported recently.[5] The sample is excited with circularly polarized light and the fluorescence intensity is measured. Fluorescence detection of circular dichroism allows one to specifically measure the circular dichroism of a fluorescent chromophore in a mixture of nonfluorescent but chiral groups. Furthermore, the total circular dichroism of the system can be determined from the concentration dependence of the fluorescence signal. The advantages and limitations of the method can be assessed better after a derivation of the equations which relate the measured signal to molecular parameters. Before doing that it is well to emphasize that this method does *not* involve the circular polarization of luminescence.[6,7] Circular polarization of luminescence gives information about excited states of a molecule; one measures the polarization of the fluorescent light. Fluorescence detection of circular dichroism gives information about the ground state of a molecule in the same way that the usual measurement of circular dichroism does.

The equations derived here apply to solutions in which there is no photoselection of the fluoresing molecules.[8,9] This requires that either (1) the direction of the transition moment for fluorescence is the same as that for absorption or (2) the fluorophore has time to rotate during the fluorescence (the rotational relaxation time is short compared to the fluorescence lifetime). If neither criterion is fulfilled the fluorescence can be measured at a particular angle[9] to obtain the fluorescence detected circular dichroism we will be describing here. Photoselection leads to the fluorescence detected circular dichroism of the oriented molecules; its discussion will be reserved for a later time.

Theory of Measurement

To understand fluorescence detected circular dichroism (FDCD) it is necessary to review first the usual circular dichroism (CD) measurement.

CD Measurement. Circularly polarized light of intensity I_0 is incident on a cell of length l containing chiral molecules. A detector (CD photomultiplier of Figure 1) measures the difference between the left and right circularly polarized transmitted light. The signal recorded is proportional to the difference between the left and right circularly polarized light divided by their sum.

$$S = K\left(\frac{I_L - I_R}{I_L + I_R}\right) \tag{1}$$

S = the signal at the recorder instrument, K = the proportionality constant dependent on photomultiplier sensitivity, amplifier gain, etc., and I_L, I_R = the transmitted intensity of left or right circularly polarized light. Use of Beer's law leads to

$$S = -K \tanh\left[\frac{2.303}{2}(A_L - A_R)\right] \tag{2}$$

A_L, A_R = the absorbance for left or right circularly polarized light. For $(A_L - A_R)$ less than 0.1 the hyperbolic tangent can be replaced by its argument; thus the signal at the recorder (the pen position on the chart) is proportional to the circular dichroism $(A_L - A_R)$. An equivalent measure of the CD is the ellipticity, θ^0.

$$\theta^0(\text{in deg}) = \frac{360}{2\pi}\frac{2.303}{4}(A_L - A_R)$$

$$\theta^0 = 32.98(A_L - A_R) \tag{3}$$

The important conclusion is that the pen position on the recorder chart is directly proportional (for $A_L - A_R \leq 0.1$ or $\theta^0 \leq 3.3°$) to the circular dichroism. As commercial CD instruments record ellipticity in degrees, θ^0, the desired relation between signal and ellipticity is

$$\theta^0 = 32.98\left(\frac{2S}{-2.303K}\right) = -28.65(S/K) \tag{4}$$

FDCD Measurement. For fluorescence detection of circular dichroism a cut-off filter absorbs incident and scattered light but transmits the fluorescence. Figure 1 shows the photomultiplier at right angles to the incident beam, but any angle can be used. The circular dichroism can only be measured at wavelengths below the cut-off wavelength. The maximum in the fluorescence is at high enough wavelength relative to the absorption so that this limitation is usually not a problem. It is important that the fluorescence from different parts of the cell be equally sampled. We assume that the measured fluorescence is integrated over the entire cell. The fluorescence signal recorded is proportional to the difference in fluorescence intensity when left and right circularly polarized light beams are incident.

$$S_F = K\left(\frac{F_L - F_R}{F_L + F_R}\right) \tag{5}$$

S_F = the signal at the recorder, K = the instrument proportionality constant, and F_L, F_R = the fluorescence intensity measured at FDCD photomultiplier when left or right circularly polarized light is incident.

For simplicity we will first consider a sample which contains only one fluorescent species. This species does not have any light energy transferred to it. Later we will generalize the

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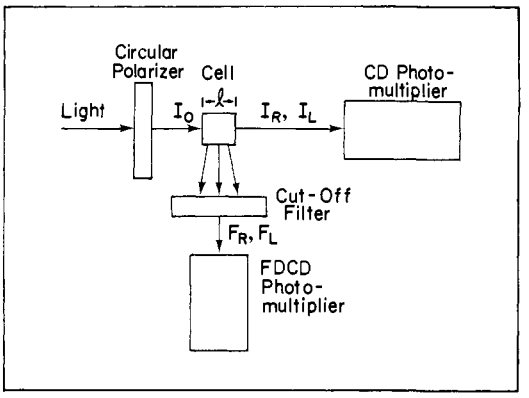

Figure 1. A diagram showing how fluorescence detection of circular dichroism (FDCD) compares with the normal method. The FDCD detector in principle can be placed at any angle relative to the incident light.

equations. The differential fluorescence intensity, dF, emitted by a differential slice of the sample at x is proportional to

$$dF = 2.303\epsilon_F c_F \phi I(x)\, dx \tag{6}$$

ϵ_F = the molar absorptivity of fluorophore with concentration c_F in mole per liter, ϕ = the quantum yield of fluorophore, and $I(x)$ = the intensity of light at position x. Integrating over the length, l, of the cell we obtain

$$F = \frac{I^0 \phi \epsilon_F c_F l(1 - 10^{-A})}{A} \tag{7}$$

A = the absorbance of sample in the cell. It includes absorbance of solvent, fluorophore, and other nonfluorescent species. Substitution into eq 5 gives

$$S_F = K\left[\frac{\epsilon_{FL}A_R(1 - 10^{-A_L}) - \epsilon_{FR}A_L(1 - 10^{-A_R})}{\epsilon_{FL}A_R(1 - 10^{-A_L}) + \epsilon_{FR}A_L(1 - 10^{-A_R})}\right] \tag{8}$$

We have clearly assumed that the quantum yield is independent of the circular polarization of the exciting light; this is generally true. Equation 8 corresponds to eq 2 of ref 1. We note that in eq 2 of ref 1 the parameter S should have been defined as the measured ellipticity divided by 33 (2/2.3) instead of 33. Rearranging eq 8

$$S_F = K\left[\frac{(\Delta\epsilon_F/\epsilon_F) - 2R}{2 - (\Delta\epsilon_F/\epsilon_F)R}\right] \tag{9}$$

$$R = \frac{A_L(1 - 10^{-A_R}) - A_R(1 - 10^{-A_L})}{A_L(1 - 10^{-A_R}) + A_R(1 - 10^{-A_L})}$$

$\Delta\epsilon_F = \epsilon_{FL} - \epsilon_{FR}$ the molar circular dichroism of the fluorophore, and ϵ_F = the molar extinction coefficient of the fluorophore. For $(A_L - A_R) \leq 0.1$ and $(A_L - A_R)/A \leq 0.1$, R can be simplified considerably to

$$R_I = \frac{\Delta A}{2A} - \frac{2.303\Delta A 10^{-A}}{2(1 - 10^{-A})} \tag{10}$$

$\Delta A = A_L - A_R$ = the circular dichroism of the sample. The value of R_I is equal to R within 0.3% for ΔA and $\Delta A/A \leq 0.1$; the approximation involved in eq 10 is no worse than the one routinely used in the normal CD measurement. Assuming further that $\Delta\epsilon_F/\epsilon_F \leq 0.1$ we obtain

$$S_F = \frac{K}{2}\left[\frac{\Delta\epsilon_F}{\epsilon_F} - 2R_I\right] \tag{11}$$

Equation 9 is exact, whereas eq 11 is approximate. However, the assumptions necessary to replace eq 9 by eq 11 are very conservative so eq 11 should be applicable in nearly all situations. Comparing eq 11 with eq 4 we obtain

$$\theta_F^0 = -14.32\left(\frac{\Delta\epsilon_F}{\epsilon_F} - 2R_I\right) \tag{12}$$

(for 1 fluorescent species)

This equation relates the ellipticity (in degrees) measured on the chart paper of the recorder to the fluorescence detected circular dichroism. There are two contributions. One is the Kuhn dissymmetry factor, $g_F = \Delta\epsilon_F/\epsilon_F$, of the fluorophore which is independent of concentration. The other is R_I which depends on concentration and is proportional to the circular dichroism of the sample. The first term thus measures the molar circular dichroism of the fluorescent species only, whereas the second term measures the circular dichroism of all the species in the sample. The two terms can be determined separately by various methods. One method is to measure θ_F^0 vs. total concentration and extrapolate to infinite dilution. Expansion of R_I as ΔA and A approach zero shows that at low concentration R_I approaches $(2.303/2)\Delta A$. As $\Delta A = \Delta\epsilon lc$ we can write

$$\theta_F^0 = -14.32\left(\frac{\Delta\epsilon_F}{\epsilon_F} - 2.303\Delta\epsilon lc\right) \tag{13}$$

From a plot of θ_F^0 vs. c the dissymmetry factor, $\Delta\epsilon_F/\epsilon_F$, for the fluorophore is obtained from the intercept and the total circular dichroism, $\Delta\epsilon$, is obtained from the limiting slope. Another method is simply to measure R_I in a separate experiment by measuring ΔA and A. This latter method is necessary when the species in the sample are concentration dependent as in associating systems.

Fluorescence detection gives the Kuhn dissymmetry factor $\Delta\epsilon_F/\epsilon_F$ as the experimentally measurable quantity. In principle (eq 13) an extrapolation must be used to obtain $(\Delta\epsilon_F/\epsilon_F)$ from the measured θ_F^0. In practice the sensitivity of the fluorescence measurement often makes the concentration-dependent term negligible so that θ_F^0 depends only on $(\Delta\epsilon_F/\epsilon_F)$. Alternatively, the fluorescence sensitivity can be utilized by adding a small amount of a nonchiral and noninteracting fluorophore to a sample whose circular dichroism is of interest. The fluorophore now acts simply as a reporter molecule. The $\Delta\epsilon$ obtained from the slope of θ_F^0 vs. c (eq 13) is just the circular dichroism of the sample.

Special Cases. Equation 12 is our general equation, but it can be modified in various ways to explicitly apply to special cases.

1. Only one absorbing species is present. The sample contains only one solute species and the solvent absorption is negligible. Then $(\Delta A/A) = (\Delta\epsilon_F/\epsilon_F)$, therefore

$$\theta_F^0 = \frac{-32.98\Delta A 10^{-A}}{1 - 10^{-A}} \quad \text{(for 1 species only)} \tag{14}$$

To obtain the CD of the one species the absorption must be measured in a separate experiment. In the limit as concentration becomes zero, eq 14 gives

$$\theta_F^0 = -14.32\left(\frac{\Delta A}{A}\right) = -14.32\left(\frac{\Delta\epsilon_F}{\epsilon_F}\right) \tag{15}$$

(for 1 species only)

This result agrees with eq 13 as it must.

2. More than one fluorescent species is present. Equation 12 can be easily generalized if there are several fluorescent species with similar fluorescence spectra.

$$\theta_F^0 = -14.32\left[\frac{\Sigma_i \phi_i c_i \Delta\epsilon_i}{\Sigma_i \phi_i c_i \epsilon_i} - 2R_I\right] \tag{16}$$

$\Delta\epsilon_i$ = the molar CD of species i, ϵ_i = the molar absorptivity of species i, ϕ_i = the quantum yield of species i, and c_i = the concentration in mole per liter of species i. The different fluorescent species could be different conformations of a single molecule, or groups at different sites in a macromolecule, or mixtures of several homologues, etc. We note that the contribution of each species to the FDCD is weighted by its quantum yield. To get a large FDCD signal we need both a large quan-

tum yield and a large circular dichroism. Species which fluoresce strongly but have a small CD will decrease the FDCD signal. Measurement of the FDCD, the CD, and the absorbance of systems with more than one fluorescent species should be quite informative. The FDCD measurement gives the quantum yield weighted ratio of CD and absorbance (eq 16), whereas the usual measurements lead to the unweighted ratio,

$$\Sigma_i c_i \Delta\epsilon_i / \Sigma_i c_i \epsilon_i$$

3. Energy transfer. When the species which emits the fluorescent light is not the same one which absorbs the incident light, eq 16, must be slightly modified. The probability of transfer of energy from species j to i must be included. Thus, in eq 16 $\Delta\epsilon_i$ and ϵ_i must be replaced by

$$\Delta\epsilon_i + \sum_{j \neq i} a_{ji} \Delta\epsilon_j c_j / c_i$$
$$\epsilon_i + \sum_{j \neq i} a_{ji} \epsilon_j c_j / c_i \qquad (17)$$

a_{ji} = the average probability of transfer of energy from species j to species i. The value of a_{ji} will in general depend on the concentrations of i and j in addition to the other properties of the system. We have assumed that the quantum yield of fluorescence is independent of whether the excitation was obtained by direct absorption or by transfer. An effective transfer coefficient, a_{ji}, should be used to be consistent with this notation. The important point to note is that FDCD measures the CD of fluorescent species plus any species which transfer energy to a fluorescent species. One can think of the product $\phi_i a_{ji} c_i^{-1}$ as an effective quantum yield for each species j and just use eq 16 to include energy transfer. It does not matter which species emits the light; it is only important that a certain fraction of absorbed light is emitted. We do assume, however, that the absorbed energy is not transferred over distances of the same order of magnitude as the length of the cell.

Signal to Noise. The optimum signal to noise for the measurement of CD[10] (for a shot noise limited signal) occurs for a sample absorbance of $A = (2/\ln 10) = 0.87$. For shot noise limited measurement of FDCD we find that the optimum signal to noise occurs as absorbance approaches zero. The FDCD signal is proportional to $(F_L - F_R)$. Shot noise is proportional to the square root of the average signal, $(F_L + F_R)^{1/2}$. The ratio of this FDCD signal to shot noise is a maximum at absorbance $A = 0$. Of course both $(F_L - F_R)$ and $(F_L + F_R)^{1/2}$ approach zero as absorbance approaches zero, but their ratio is finite. In practice as the concentration of the fluorescing species becomes zero, noise from sources other than shot noise becomes important. The practical optimum signal to noise is then the lowest sample absorbance for which shot noise still predominates.

Summary. Measurement of the fluorescence signal, θ_F^0, obtained from commercial circular dichrographs leads to the dissymmetry factor $(\Delta\epsilon_F/\epsilon_F)$ for fluorophores. If the measurement is made in dilute enough solution so that the CD of the sample is negligible, then

$$\Delta\epsilon_F/\epsilon_F = -0.0698\theta_F^0 \qquad (18)$$

The $\Delta\epsilon_F$ and ϵ_F must be interpreted as the molar CD and molar absorptivity of species which fluoresce directly or which transfer energy to fluorescent molecules. For mixtures, $\Delta\epsilon_F$ and ϵ_F are quantum yield weighted averages (eq 16).

Although the dissymmetry factor is a useful measure of chirality and conformation it may be of interest to obtain $\Delta\epsilon_F$ directly. For systems containing a single fluorescent species, ϵ_F and therefore $\Delta\epsilon_F$ are easily obtained. For a fluorescent chromophore which is part of a macromolecule, obtaining $\Delta\epsilon_F$ is more difficult. A wavelength may be found where the macromolecular contribution to the absorption is negligible, or a simple model compound may be used to estimate a value of ϵ_F at a single wavelength. Once one value of ϵ_F is known (or assumed), the absorption spectrum can be approximated from a measured fluorescence activation spectrum.

FDCD and Molecular Structure. Interpretation of the FDCD is similar to interpretation of the usual CD spectra. The only difference is that in FDCD a transition must lead to fluorescence in order to be detected. This means that only electrically allowed transitions should contribute significantly to the FDCD. Magnetically allowed but electrically forbidden transitions such as the $n\pi^*$ in carbonyls would be expected to have very small quantum yields for fluorescence. Furthermore, although many electrically allowed transitions have small quantum yields, they can transfer energy to a fluorophore. The electrically forbidden transitions also lack this route to fluorescence emission.

The first step in interpreting an FDCD spectrum is to assign regions of the spectrum to fluorescent transitions in the system. For a single fluorophore the $\Delta\epsilon_F/\epsilon_F$ spectrum will have an approximately flat region for each transition, because in the wavelength region corresponding to a single transition $0 \rightarrow A$

$$\frac{\Delta\epsilon_F}{\epsilon_F} = \frac{4R_{0A}}{D_{0A}} = \frac{4\mathrm{Im}\boldsymbol{\mu}_{0A} \cdot \mathbf{m}_{A0}}{\boldsymbol{\mu}_{0A} \cdot \boldsymbol{\mu}_{0A}} \qquad (19)$$

where R_{0A} is the rotational strength of the fluorescent transition and D_{0A} is the dipole strength.[11] Im means the imaginary part and $\boldsymbol{\mu}_{A0}$ and $\mathbf{m}_{A0}$ are the electric and magnetic dipole transition moments. All the standard methods for relating rotational and dipole strengths to molecular structure can be used. Overlapping transitions in one fluorophore or a polymer with many fluorescent groups (or energy transfer from many transitions) will have a spectrum which is more complicated. There will be a sum of rotational and dipole strengths contributing to $\Delta\epsilon_F$ and ϵ_F at each wavelength.

$$\frac{\Delta\epsilon_F}{\epsilon_F} = \frac{4\Sigma_A \phi_A R_{0A} f_A{}^R(\lambda)}{\Sigma_A \phi_A D_{0A} f_A{}^D(\lambda)} \qquad (20)$$

The functions $f_A{}^R(\lambda)$ and $f_A{}^D(\lambda)$ are the shapes of the CD and absorption band for transition A; they are similar but not necessarily identical.[12]

Macromolecules. We will mention two useful methods for learning the structure from the FDCD. First-order perturbation gives a simple method for obtaining qualitative, or semiquantitative, information about conformation near a fluorophore. To first order in the interaction potential, the equation for transition from $0 \rightarrow a$ in fluorophore i is[13]

$$\frac{4R_{0A}}{D_{0A}} = \frac{2\pi\nu_{0a}}{\mu_{i0a}{}^2 c} \sum_{j \neq i} \sum_{b \neq a} \nu_{0b} \frac{V_{i0a;j0b} \mathbf{R}_{ij} \cdot \boldsymbol{\mu}_{i0a} \times \boldsymbol{\mu}_{j0b}}{h(\nu_{0b}{}^2 - \nu_{0a}{}^2)} \qquad (21)$$

The FDCD spectrum thus depends on how a fluorescent transition at frequency ν_{0a} with transition dipole moment $\boldsymbol{\mu}_{0a}$ interacts with all transitions $(\nu_{0b}, \boldsymbol{\mu}_{j0b})$ on neighboring groups at positions $\mathbf{R}_{ij}$. The interaction energy $V_{i0a;j0b}$ between the transitions is usually approximated by interactions between transition monopoles or dipoles; it will decrease with distance at least as fast as R_{ij}^{-3}. In eq 21 we have chosen the chromophore to have no intrinsic CD and we have ignored static field effects. That is, we have chosen μ's and ν's to correspond to the groups in the static field of the rest of the molecule. We reemphasize that all terms involving higher powers of V_{ij} have been dropped in eq 21. If there were more than one fluorescent transition we would need to sum over i and weight each transition by its quantum yield, ϕ_i. All-order perturbation methods[14] are available for more accurate calculations of R_{0a} and D_{0A}.

An all-order polarizability method can be used to calculate

$\Delta\epsilon_F/\epsilon_F$ directly as a function of wavelength.[15] This method has been successful in CD calculations of polynucleotides.[16] The equation is

$$\frac{\Delta\epsilon_F}{\epsilon_F} = -\frac{2\pi}{\lambda}\frac{\sum_i \sum_{j\neq i} \phi_i \mathrm{Im} A_{ij} \mathbf{R}_{ij} \cdot \mathbf{e}_i \times \mathbf{e}_j}{\sum_i \sum_{j\neq i} \phi_i \mathrm{Im} A_{ij} \mathbf{e}_i \cdot \mathbf{e}_j} \qquad (22)$$

$$A_{ij} = \left[\frac{\delta_{ij}}{\alpha_i(\lambda)} + G_{ij}\right]^{-1}$$

where λ is the wavelength and $\mathbf{R}_{ij}$ is the distance between groups i and j. A_{ij} is the inverse of a matrix which has wavelength dependent complex polarizabilities, $\alpha_i(\lambda)$, on the diagonal and interaction terms ($G_{ij} = V_{ij}/|\mu_i||\mu_j|$) off the diagonal. Unit vectors $\mathbf{e}_i$ and $\mathbf{e}_j$ specify the directions of the one-dimensional polarizability tensors corresponding to each transition. The real and imaginary parts of the polarizability correspond to the refraction and absorption of the transition. Note that each transition is weighted by its effective fluorescence quantum yield, ϕ_i. The effective quantum yield characterizes the relative contribution of each transition to the measured fluorescence. It can take into account energy transfer and different efficiencies of detection for different fluorophores in the system.

The fluorescence quantum yield weighting of the transitions is particularly interesting for nonrigid molecules. The usual CD measures the average over all conformations; FDCD measures the average over fluorescent conformations. Comparison of the two measurements will allow a better assessment of what conformations are present in the molecules.

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Studies in the Chiroptical Properties of Selenoamino Acids[1]

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Abstract: The circular dichroism (CD) spectra of four selenium-containing amino acids were investigated and compared with those of their sulfur and methylene analogues. In the region 190–250 nm, positive Cotton effects of carboxyl and selenide (or sulfide) chromophores always correlate with L (= *S*) or L,L (= *S,S*) absolute configurations. The additive nature of such Cotton effects was demonstrated by a "difference curve" method, which permits the chiroptical properties of the Se (or S) chromophore to be determined. Results suggest that the C–Se–C selenide chromophore has three optically active transitions in the near uv range at approximately 225, 210, and 195–200 nm. The absolute configuration of natural (+)-selenocystathionine has been established spectroscopically to be L,L (= *S,S*), in agreement with enzymatic results.

Selenoamino acids first attracted interest during the latter part of the 1930's when it was shown that a cattle disease known as "alkali disease", found in parts of the United States, was closely connected with the selenium content of the soil, that plants were capable of accumulating selenium, and that the selenium so incorporated occurred in the protein fraction of the plants.[3,4] Selenoamino acids were isolated from the plant material[5] and proved to be highly toxic.[6]

These findings led to the synthesis of selenoamino acids by Fredga[7] and by Zdansky.[8] Walter and Roy[9] have reviewed the selenopeptides and selenoproteins, an undiscovered territory only a decade ago.

Optically active selenocystathionine (**2**) has been isolated from plant material[10,11] and was found to possess biological effects. Its absolute configuration was assigned as L on the basis of the lack of reactivity with D-amino acid oxidase.[11] However, the selenium compound could have inhibited the enzyme.

Since no chiroptical studies of selenoamino acids have been made, we decided to investigate this subject. Among the physical methods used for obtaining information on chiroptical properties, the technique of circular dichroism (CD) measurement is the most efficient since it gives precise data on the chirality of specific transitions. The acids under investigation contain two chromophores which absorb in the near-uv region: COOH and C–Se–C. References on simple carboxyl absorption indicate that the n $\rightarrow$ π^* transition occurs at approximately 210 nm and a $\pi \rightarrow \pi^*$ transition below 190 nm.[12] However, few literature references exist for the C–Se–C selenide chromophore. The solution spectrum (hexane) of diethylselenide[13] reveals an absorption band at 250 nm (ϵ_{max} 50). In the similarly constituted dialkyl sulfides, the C–S–C sulfide chromophore has recently been shown[14,15] to have several electronic transitions between 198 and 255 nm. The n $\rightarrow$ σ^* transitions of the C–Se–C chromophore may therefore be