Single-Molecule Orientations in Dyed Salt Crystals

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We present single-molecule confocal microscopy studies of orientational distributions for luminophores isolated in potassium hydrogen phthalate (KAP) crystals. The incorporation of dye molecules that bear no size or shape similarity to the host ions is observed, demonstrating that single-molecule studies on mixed crystals need not be restricted to isomorphous host/guest pairs. Violamine R is oriented and overgrown by the fast vicinal slopes of growth hillocks within the symmetry-related {010} growth sectors and DCM deposits in the $\{11\bar{1}\}$ growth sectors of KAP. Both mixed crystals exhibit modest absorption dichroism relative to basic pyranine-doped K_2SO_4. The latter was studied to ensure that a range of orientations was sampled in our experiments. Average orientations determined at the single-molecule level were in close agreement to ensemble-averaged measurements for all three systems, and the chromophore orientational distributions were broader than anticipated, indicating that the crystals incorporate guest molecules in a range of orientations outside the measured ensemble average.

Introduction

Single-molecule spectroscopy was pioneered by the groups of Moerner and Orrit, who studied mixed crystals of *p*-terphenyl containing pentacene, an isomorphous host/guest pair.[1−4] The condition of isomorphism, that the host and guest molecules must resemble one another in size and shape, is often a presumed prerequisite for mixed crystal growth. In this case, pentacene molecules, long hydrocarbons with an aspect ratio comparable to *p*-terphenyl, can comprehensively substitute into the *p*-terphenyl crystal lattice. Hidden heterogeneities in the spectral diffusion of single pentacene molecules within the transparent crystal host at 1.5 K were observed, establishing the utility of single molecules as "nanoreporters" of local environments in condensed media.[5] Since then, single-molecule studies have been extended to polymer hosts, glasses, surfaces, and solutions.[6−11] Yet, investigations on mixed crystal systems have so far been limited to the regime where host and guest molecules are isomorphous.[12,13] While the principle of isomorphism requires hosts and guests of similar size and shape, many counter-examples exist. Under conditions of supersaturation, solutions of simple transparent substances will frequently deposit crystals that have oriented and overgrown a wide variety of chromophores.

We previously described the use of salts as matrixes for the study of organic dye molecules in uniquely well-defined environments.[14,15] In particular, single crystals of potassium hydrogen phthalate (KAP)[16] and potassium sulfate (K_2SO_4)[17] are easily grown from aqueous solution at room temperature in the presence of many luminophores. When organic "impurities" such as dye molecules are included in crystal growth solutions of many simple transparent salts including KAP and K_2SO_4, the impurities are differentially adsorbed and overgrown by faces of the crystal not related by symmetry, a phenomenon called *inter*sectoral zoning, illustrated by DCM (4-dicyanomethylene-2-methyl-6-*p*-dimethylaminostyryl-4H-pyran) (**2**) in the $\{11\bar{1}\}$ growth sector of KAP. *Intra*sectoral zoning is observed when dye impurities inhomogeneously deposit *within* a single growth sector.[18] For instance, in the low supersaturation regime, the KAP crystal surface propagates through screw dislocations that produce growth hillocks, shallow stepped pyramids with single or multiple dislocations at the apex. When a dye expresses different affinities for vicinal regions, the result is an intrasectorally zoned dye inclusion, depicted in Figure 1. Violamine R (**1**) recognizes the {010} growth sector of KAP (i.e., intersectoral zoning) and within this volume only the fast slopes of growth hillocks (i.e., intrasectoral zoning).[19] These results express the highly specific deposition of dyes into a crystal host and show that dye inclusion crystals provide opportunities to study chromophore alignment.

From measurements of bulk dichroism, it is apparent that single-crystal matrix isolation provides an efficient orientation mechanism for included dyes. However, the degree of alignment has not been studied at the single-molecule level. Characterizing the orientational distributions of chromophores within a single-crystal matrix can reveal the extent to which overgrowth by the crystal lattice results in chromophore alignment. Here, we present single-molecule investigations of mixed salt crystals, grown from aqueous solutions at room temperature, unrestricted by host/guest isomorphism. We describe the orientational distributions of individual chromphores in dye-doped salt crystals at the limit where interluminophore interactions are negligible, using confocal fluorescence microscopy.[20−23] KAP crystals (space group $Pca2_1$, $a = 9.614$ Å, $b = 13.330$ Å, $c = 6.479$ Å)[24] were chosen as the primary host for this study because they are easily grown from aqueous solution as large {010} plates, with perfect cleavage parallel to these faces. The organic dyes **1** and **2** were used as single-molecule reporters in KAP. Highly dichroic crystals of K_2SO_4 doped with the basic form of pyranine (8-hydroxypyrene-1,3,6-trisulfonic acid) (**3**) were also studied to ensure that a variety of orientational populations was sampled.[17] A comparison is made of the ensemble-averaged and single-molecule orientations in dye inclusion crystals of KAP/**1**, KAP/**2**, and K_2SO_4/**3** in an effort

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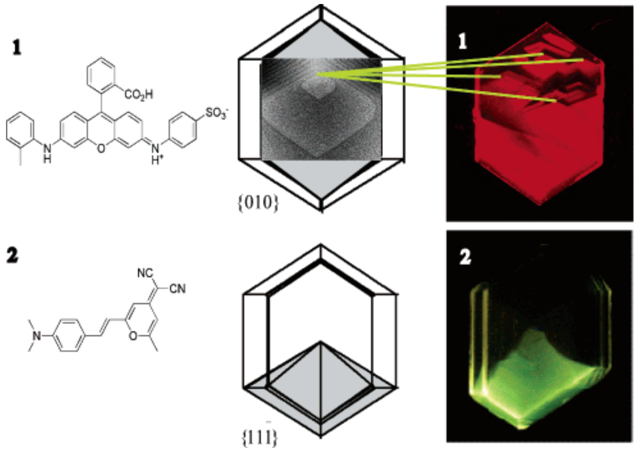

Figure 1. Dye-inclusion KAP crystals. Violamine R (**1**) is included on the fast slopes of growth hillocks within the {010} growth sector of KAP. An atomic force micrograph of KAP hillocks is represented within an idealized {010} inclusion to show the relationship between screw dislocations and dyed hillocks in the luminescence photograph. DCM (**2**) is incorporated into the $\{11\bar{1}\}$ sector of KAP.

to interrogate the efficiency of crystal growth from solution as a chromophore alignment mechanism.

Experimental Section

1 was purchased as the sodium salt from Aldrich. **3** was formed by dissolving pyranine (Kodak) in a 10^{-2} M KOH solution. Dye-inclusion crystals were grown by evaporation in a 500-mL solution containing either KAP or K_2SO_4 (Mallinckrodt), and the dye of interest was dissolved in deionized water (Barnsted NANOpure, 18.2 MΩ cm^{-1}). **2** (Aldrich) was dissolved in 10 mL ethanol before addition to the growth solution. Evaporation was performed in a temperature-controlled chamber held at 30 ± 0.1 °C. Heavily dyed single crystals of KAP/**1**, KAP/**2**, and K_2SO_4/**3** were grown from evaporation of aqueous solutions containing 10^{-4} M dye. Corresponding samples with densities suitable for single-molecule investigations were grown from 10^{-8} M dye solutions.

Heavily dyed crystals were characterized by polarized absorption and fluorescence microscopy. The segregation coefficient (σ_{seg}), the mole ratio of dye in the crystal to dye in the growth solution, was determined by absorbance measurements on redissolved crystals. Solution and crystal absorption spectra were obtained with a microabsorption spectrometer consisting of a polarizing microscope (Olympus BX-50) coupled to a UV−vis spectrophotometer (Si-Photonics 440). The extinction directions of the birefringent samples were used to orient the sample relative to input polarization. Corresponding bulk fluorescence measurements were recorded with a fluorimeter (SPEX FluoroMax-2) employing excitation at 532, 488, and 464 nm for crystal samples of KAP/**1**, KAP/**2**, and K_2SO_4/**3**, respectively.

Single-molecule studies were performed using an inverted confocal microscope (Nikon, TE2000-U). Dyed crystals were excited with low power (<10 μW) illumination from a 532-nm or 488-nm solid-state laser (NovaLux, Protera) that was filtered (Chroma) and focused to a diffraction-limited spot with a 100× oil-immersion objective (Nikon, PlanFluor 1.3 NA). The excitation polarization was adjusted using a half-wave plate (CVI Laser). Crystals were attached to glass coverslips (Corning) with vacuum grease and were mounted in an inverted orientation on a closed loop $x-y$ piezo scanning stage (Queensgate, NPS-XY-100B) to avoid imaging through the glass. Immersion oil (Zeiss, $n = 1.518$) was used for index matching. Emission from the sample was spectrally filtered using the appropriate dichroic mirror and emission filters (Chroma, 500-nm long-pass filters for 488-nm excitation or 600 ± 50-nm band-pass filter for 532-nm excitation) and was spatially filtered with a confocal pinhole (CVI, 50 μm). Total emission intensity was measured over a 10×10 μm^2 area, using a 100-nm step size and 100-ms integration time per point. Emission was detected using a single-photon-counting avalanche photodiode (PerkinElmer, SPCM-AQR-16). Data collection was systematized with Labview (National Instruments). The existence of single molecules was verified by observing blinking dynamics followed by complete photobleaching and by a measured fluorescence image size that was close to the diffraction limit (~250 nm). The single-molecule orientations (θ) were determined relative to the host lattice by measuring the polarized excitation anisotropy, obtained in subsequent scans of the total fluorescence intensity following excitation along orthogonal eigenmodes of the samples using

$$\theta = \tan^{-1}\sqrt{(I_{\parallel}/I_{\perp})}.$$

However, with a high NA objective that has a large half-aperture angle ($\theta_{obj} \approx 60°$), using the aforementioned expression for θ can be problematic, since it has been established that z-mixing of the excitation polarization precludes measurement of the orientational extremes.[25] The effect of high NA on the measured orientations was examined by repeating single-molecule experiments on KAP/**1** using a 0.8-NA objective (Nikon, Fluor adjustable 1.3-0.7 NA), and the orientational results obtained at low NA were unchanged from those reported here. Furthermore, using a high NA objective motivated the inclusion of highly dichroic K_2SO_4/**3** crystals in our analysis to determine if the ensemble-averaged information was recovered as the orientational threshold was approached. A correction was made for the strong birefringence of KAP ($\Delta n_{c-a} = 0.161$), which disproportionately changes the effective electric field experienced by molecules when illuminated along the crystal's eigenmodes, using the Lorentz approximation as previously described.[26] The intrinsic error in θ (the orientation of an individual dye molecule relative to the host lattice), determined by the emission fluctuations of a single-molecule time-trace, was approximately ±5°.

Results and Discussion

Violamine R (1) Dyed KAP. The absorption maximum of **1** is 525 nm in water and 544 nm in the crystal ($\sigma_{seg} = 0.013$), with fluorescence maxima of 570 and 609 nm, respectively. The average orientation of the absorption dipole moment in heavily dyed KAP/**1** crystals was found by polarized absorption and excitation measurements to be 44.5° and 45.0° from [100] in the ac-plane, respectively. To develop a molecular-level understanding of how this rhodamine derivative is oriented in the KAP crystal host, single-molecule confocal fluorescence measurements were performed. Figure 2 shows a typical data set, consisting of false-color images of the fluorescence from KAP/**1** grown from a solution containing 10^{-8} M dye, with images corresponding to excitation along the [100] and [001] eigenmodes of the crystal. The distance between dye molecules is consistent with the measured σ_{seg} for the crystal. Given that only ~1% of dye in the growth solution is incorporated into the crystal, molecules of **1** should be ~12.4 μm apart in the {010} subvolume, in agreement with confocal images; the average interluminophore distance was ~4 μm. Molecules that are brighter with excitation polarization parallel to [100] and [001] have absorption dipole moments oriented closer to the a-

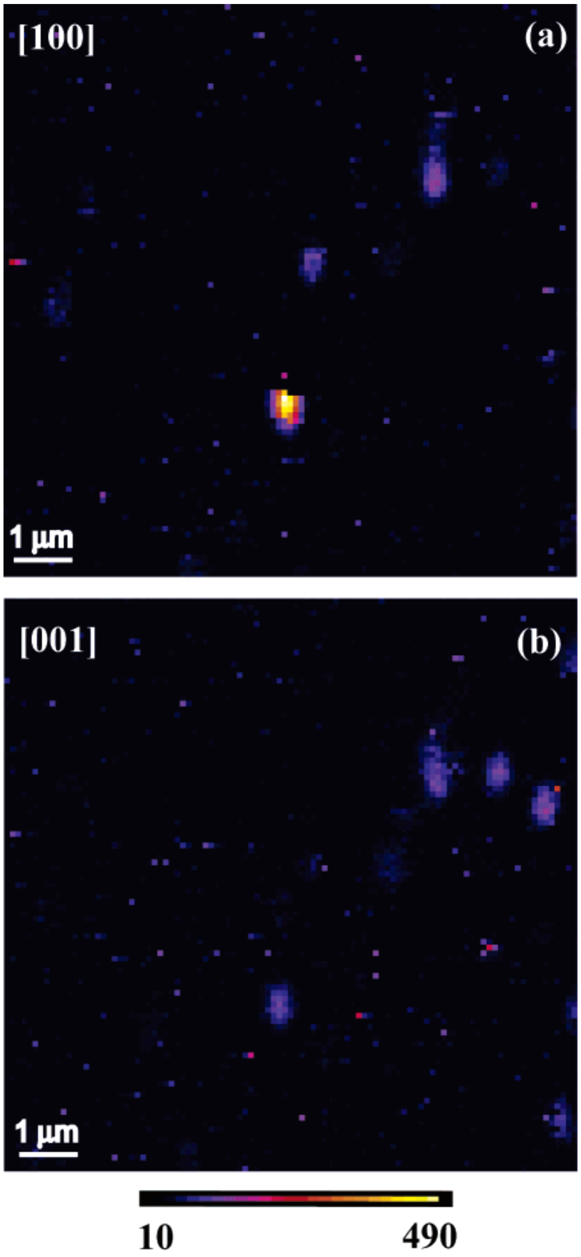

Figure 2. False-color 10 × 10 μm^2 fluorescence images from a KAP crystal grown from 10^{-8} M **1**, obtained from 532-nm excitation along orthogonal eigenmodes (a) [100] and (b) [001]. Color scale corresponds to counts per 100 ms.

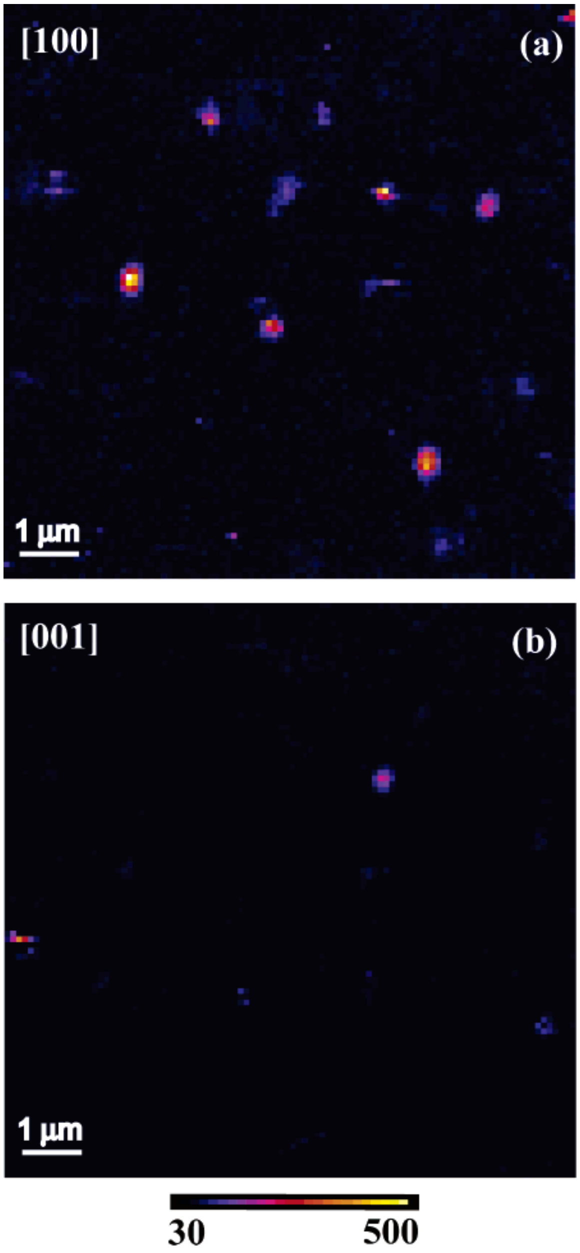

Figure 3. False-color 10 × 10 μm^2 fluorescence images from a KAP crystal grown from 10^{-8} M **2**, obtained from 488-nm excitation along (a) [100] and (b) [001]. Color scale corresponds to counts per 100 ms.

and *c*-direction, respectively. The signal-to-noise ratio (SNR) of the brightest molecules was approximately 10:1. Those molecules for which the SNR was greater than 3 were included in the orientational analysis. From the measured polarized absorption dichroism in several crystal samples, the orientations for 108 individual molecules were determined. An average value of 42.3 ± 10.4° (standard deviation of the distribution) from [100] was obtained (Figure 4), in close agreement with the aforementioned ensemble-averaged dichroism. However, the single-molecule measurements demonstrate that the orientational distribution is broad, with angular orientations ranging from 20.1° to 72.9° relative to [100]. From the experiments using a low NA objective, the orientations of 68 molecules were compiled to give an average value of 46.9 ± 14.9°, with orientational extremes at 11.1° and 78.9° from [100]. The mean values are statistically equivalent for the experiments using both high and low NA objectives; the histograms are similarly wide. Thus, the broad orientational distribution observed at high NA is a real consequence of mixed crystal structure. Considering the intrinsic organization present within a single crystal, and our observations of chemical zoning in heavily dyed crystals, this result is surprising. However, the absorption dipole moment of **1** is highly dependent on the molecular conformation.

DCM (2) Dyed KAP. To evaluate the influence of molecular architecture on orientational distribution**,** we studied DCM, a widely investigated laser dye and archetypal NLO chromophore[27] that contains a dimethylamino electron donor linked to a dicyanomethylene electron acceptor by a conjugated bridge. The “donor−bridge−acceptor” geometry of DCM enables intramolecular charge transfer (CT). Upon photoexcitation, the excited DCM molecule is promoted to the locally excited (LE) state (S_1) and may undergo either trans-cis isomerization or CT from the dimethylamino donor to the dicyanomethylene acceptor, where it is thought that the dimethylamine groups twists by 90° to produce the most favored charge-separated geometry,

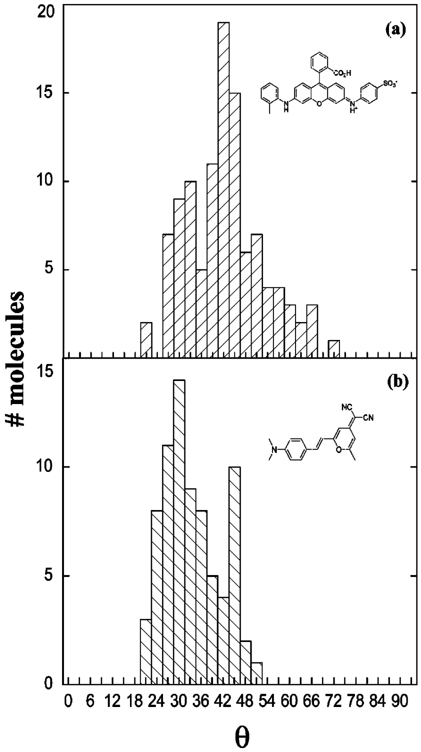

Figure 4. Orientational distribution histograms (bin size = 3°) of single molecules in dyed KAP. (a) Histogram of chromophore orientation for 108 violamine R molecules, measured as an angle between 0° and 90° from [100]. The average orientation was 42.3 ± 10.4° from [100]. (b) Orientational distribution for 75 DCM molecules; the average was 35.3 ± 7.4° from [100]. Orientations were determined at 1.3 NA.

the so-called twisted intramolecular charge-transfer (TICT) state (S_2).[28−34] Prior work has shown that the excited state relaxation is largely dependent on solvent polarity, since the charge-separated geometry is stabilized in polar solvents, thereby promoting the S_2-CT state and lowering its energy.[32] Furthermore, in nonpolar solvents, deactivation of the S_1-LE state is primarily driven by internal conversion and not through states involving significant changes in molecular structure such as trans-cis isomerization and CT formation.[33] Recently, dual emission of DCM was observed when incorporated into the zeolite MCM-41, an organized medium with moderate polarity.[34]

The absorption maximum of DCM is 492 nm in ethanol and 467 nm in saturated KAP solution, with fluorescence maxima of 595 and 589 nm, respectively. The heavily dyed KAP/**2** crystal ($\sigma_{\text{seg}} = 0.021$) exhibits a visible absorbance spectrum with maxima at 408, 432, and 460 nm and emission at 470, 507, and 545 nm. The fluorescence profile of the dyed crystal demonstrates a hypsochromic shift, relative to solution. It is possible that electrostatic interactions between DCM and its nearest neighbors in the KAP host may account for the observed hypsochromic shift. However, semiempirical calculations[35] were unable to reproduce the absorbance spectrum of KAP/**2** when considering the chromophore in close contact (<3 Å) with a single ion pair in the lattice. An alternative explanation is that the KAP lattice restricts the DCM chromophore to an energetically unfavorable geometry, relative to the gas phase, thereby increasing its electronic excitation energies. This explanation is similar to the mechanism proposed by Guo et al. for the hypsochromic shift of emission observed in DCM-doped zeolites.[34]

At the laser excitation wavelength employed (488 nm), the absorption and polarized excitation dichroism in heavily dyed KAP/**2** revealed an average dye orientation of 39.3° and 31.0° from [100], respectively. Figure 3 presents false-color images of the total emission from a lightly loaded single crystal with excitation along the [100] and [001] eigenmodes. The observed intramolecular distance between dyes is in agreement with the measured σ_{seg}. The orientations for 75 molecules of **2** in KAP were calculated from the polarized excitation anisotropy, and the average was determined to be 31.9 ± 7.8° from [100], in agreement with bulk measurements. However, DCM also exhibits a wide range of chromophore orientations in the crystal, from 20.8° to 50.7° from [100]. The observed fluorescence time traces from single molecules (not shown) indicate that dual emission is exhibited from DCM in the crystal host. Dual emission suggests that chromophores are incorporated into the host matrix in more than one molecular geometry. In an attempt to model the orientational energetics of DCM included in KAP, we used the AM1 method[35] to optimize the structure of a chromophore within a fixed crystal lattice. First, we constructed holes in a 3 × 3 KAP lattice by removing a pair of potassium and phthalate ions, which comprises the smallest hole (415.2 Å^3) that a DCM molecule (417.9 Å^3) might occupy. The chromophore was inserted into the vacancy and was relaxed in response to the fixed KAP lattice. It was unreasonably twisted. A reasonable model was constructed by substituting DCM for three ion pairs[36] in the KAP lattice so that the projection of the DCM long axis was ~35° from [100] in the *ac*-plane. Figure 5 shows that the elongated occupiable volume created by vacating this area is aligned with the $\{11\bar{1}\}$ surfaces. Hence, chromophores may incorporate into the $\{11\bar{1}\}$ growth sector by adsorbing along the growing step edge, and the projection of the molecular dipole-moment orientation on the *ac*-plane would be approximately 34°, in agreement with our observations. Single-molecule measurements confirm that other geometries are manifest.

Basic Pyranine (3) Dyed K_2SO_4. Given that the dichroic ratios of KAP/**1** and KAP/**2** are similar, highly dichroic dyed single crystals of K_2SO_4/**3** were investigated. The absorption maximum of **3** is 455 nm in water and 464 nm in the crystal, with fluorescence maxima of 509 and 512 nm, respectively. The average orientation of the absorption dipole moment in heavily dyed crystals was found by polarized absorption and excitation measurements to be 22.4° and 26.2° from [001], respectively. These orientations correspond to a dichroic ratio of ~5 for [001]/[100], whereas dyed KAP crystals exhibited rather modest dichroism (≤2 for [100]/[001]).

From confocal fluorescence measurements, the orientations of 19 molecules of **3** were determined, revealing an average orientation of 27.5 ± 6.6° from [001]. Therefore, single-molecule measurements validate the aforementioned ensemble-averaged values but again demonstrate that the distribution of chromophore orientations is broader than expected, ranging from 16.1° to 41.2° from [001]. Although prior work on this system[17] suggested a dichroic ratio greater than 10 for [001]/[100], and an average transition dipole moment orientation nearly overlapping the *c*-axis of K_2SO_4, our ratios were smaller (both bulk and single molecule). Nonetheless, the absorption dichroism of K_2SO_4/**3** is sufficiently different than the dyed KAP samples to ensure sampling diversity in the single-molecule measurements.

Conclusions

We have presented single-molecule investigations on mixed crystal systems comprised of host/guest pairs far from the isomorphous regime that are easily grown at room temperature in aqueous solution. Confocal fluorescence microscopy was used to characterize the orientational distributions of mixed salt

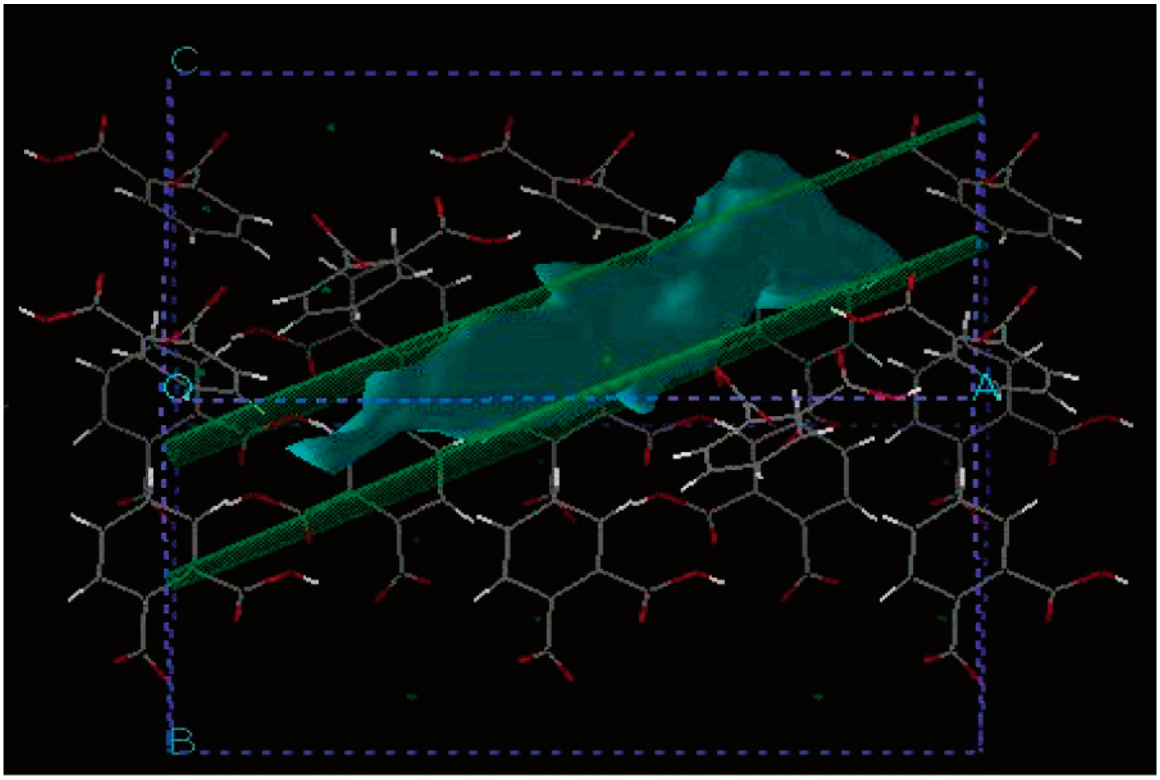

Figure 5. Model for DCM in the KAP lattice that would lead to the observed average orientation. DCM can incorporate into the occupiable volume (shown in blue), which lies within the $\{11\bar{1}\}$ facets (shown in green). Crystallographic axes (A, B, C) are labeled relative to the origin (O).

crystals of **1**- and **2**-doped KAP. For both samples, the center of the chromophore orientational distribution was in agreement with the ensemble-averaged value. For KAP/**1**, an average orientation of 42.3 ± 10.4° from [100] was obtained, with chromophore orientations ranging from 20.1° to 72.9° relative to [100]. Single crystals of KAP/**2** exhibited a sizable hypsochromic shift in absorption and emission relative to the solvated chromophore. Our data suggest that dual emission was observed when incorporated into the KAP host. The average orientation of 75 DCM molecules was determined to be 31.9 ± 7.8° from [100], with values ranging from 20.8° to 50.7° from [100]. These observations are consistent with face-on adsorption of DCM to $\{11\bar{1}\}$, further directed by steps parallel to $[\bar{1}0\bar{1}]$, the intersection of {010} and $\{11\bar{1}\}$. Studies of highly dichroic **3**-doped K_2SO_4 crystals found an average chromophore orientation of 27.5 ± 6.6° from [001], also in agreement with current bulk investigations, with values ranging from 16.1° to 41.2° from [001]. For all three of the systems studied here, single-molecule measurements revealed chromophore orientational distributions that were broader than anticipated. The breadth of the distributions for KAP/**1** and KAP/**2** were quite similar, despite the conformational rigidity of the DCM backbone relative to the conformational flexibility of **1**. Single dye molecules in modestly dichroic KAP/**1** and KAP/**2**, and highly dichroic K_2SO_4/**3**, exhibited orientations over a 52.8°, 29.9°, and 25.1° range, respectively. The observed chromophore orientational range is decreased as the dichroism of the sample is increased, as expected. This suggests that although an ensemble of dyes prefers to include within a small orientational range that is dependent on the particular host/guest system, the crystal does indeed incorporate chromophores in a variety of orientations outside the ensemble average. Overall, the results presented here highlight the molecular details hidden by conventional ensemble-averaged methods and provide insight into the extent to which dye inclusion into a crystal lattice results in chromophore alignment.

This work is also relevant to the optical characterization of dye-doped polymeric systems that are used as electrooptic (EO) switches. Currently, the translation of molecular systems with large hyperpolarizabilities to macroscopic dye-composite materials with correspondingly large EO activity is not well understood.[37] Symmetry dictates that electrooptical activity requires noncentrosymmetry, which for dye-doped polymer systems is established by applying an external electric or “poling” field across the composite material. However, recent work suggests that electric-field poling is only partially effective in achieving molecular alignment,[38] and a molecular-level understanding of chromophore ordering in dye-doped polymeric materials is crucial to extending the performance of organic molecules in NLO applications. An interesting comparison between alignment mechanisms in dyed composite materials presents itself, namely, to establish the extent to which intrinsic (crystal growth) and extrinsic (electric-field poling) in **2**-doped KAP and polymer host systems results in chromophore alignment at the single-molecule level. Such comparative studies are underway.

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