

Cooperative self-assembly of cyanines on carboxymethylamylose and other anionic scaffolds as tools for fluorescence-based biochemical sensing*

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Abstract: We recently found that certain cyanines form tight complexes with carboxymethylamylose (CMA) in aqueous solutions and that in these complexes the cyanine exists as a strongly fluorescent and stable J-aggregate. Cyanine dyes are characterized by their ability to form J-aggregates showing very narrow absorption and fluorescence spectra relative to the monomer. Although they have found uses in sensing applications, the practicability has been limited in many cases due to the low quantum efficiencies for J-aggregate fluorescence. The CMA-cyanine complex is formed by a cooperative self-assembly in which both components undergo conformational changes during the association. The CMA exists as a random coil in solution prior to complex formation; helix formation is prevented due to repulsion of the charges on the carboxymethylated glucose units. The cyanine exists as a nonfluorescent monomer in the same solutions. A helical atomic force microscopy image and large induced circular dichroism (CD) spectra of the cyanine J-aggregate indicate that the self-assembly is a superhelix scaffold of CMA decorated with J-aggregates of the cyanine. Similar behavior was also observed with carboxymethylated cellulose (CMC). Enzymatic disruption of the helical structures (e.g., by the use of amylase to disrupt the structure of CMA helix) leads to the disappearance of the J-aggregate-associated fluorescence. The photophysical behavior and applications of this complex for sensing are discussed.

Keywords: cyanine dyes; carbohydrate polymers; biosensing; fluorescence; carboxymethylamylose.

The aggregation of cyanine dyes has been widely studied in a variety of contexts, ranging from the photographic industry to the staining of deoxyribonucleic acid (DNA) [1–3]. We previously demonstrated the helicogenic property of cyanine dye that transformed chiral polymeric molecules to supra-

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helical structures accompanied by strong fluorescence (described in detail below). We now provide a novel application of this phenomenon for biological sensing.

It has been established that several different types of aggregates, characterized by spectral shifts compared to monomer and emission (or lack thereof) can be formed depending on the molecular environment, concentration, and physical state [1,4,5]. Of the various aggregate types observed, the J-aggregates, characterized by very narrow intense absorption and narrow, only slightly red-shifted fluorescence (relative to the monomer dye), have been the focus of most interest, especially for practical applications in spectral sensitization and various sensing applications [6,7]. Although J-aggregation has been observed in a variety of environments ranging from films and crystals to cavities of mesoporous particles [8] to DNA grooves [9–11], it is still unclear exactly what factors tip the balance in favor of J-aggregation for structurally similar families of cyanines.

Thus, it has been found that while certain cyanines form J-aggregates when adsorbed onto clay nanoparticles, seemingly structurally similar cyanines may exhibit H-aggregation or dimerization under the same conditions [12]. Although the J-aggregate absorption and fluorescence spectra for cyanines are quite characteristic, it has been found that the precise structure, spectral characteristics, and size may be environment-sensitive [13,14]. The sharp emission spectra of J-aggregated cyanines and the structural diversity of the cyanines have made them attractive candidates for fluorescence-based sensing applications [15]. However, in many cases their potential utility as an array of versatile fluorescent tags is attenuated by the low quantum efficiency for most J-aggregate fluorescence.

Studies over the last few years have shown that a series of polyelectrolytes with variable numbers of cationic polymer repeat units (PRU) constructed with cyanine chromophores pendant, but not conjugated, on a poly-L-lysine backbone (**1**) exhibits characteristic J-aggregate absorption and fluorescence in aqueous solution and when adsorbed onto anionic supports (Fig. 1) [15–17]. Fluorescence from such ensembles and their highly efficient quenching by energy or electron-transfer quenchers can be the basis for a variety of biosensing applications. For aqueous solutions, there was no aggregation of the monomer cyanine evident from either absorption or fluorescence [16]. There was little evidence for J-aggregation in the absorption spectra of oligomers having 6 and 33 PRU; however, with higher-molecular-weight polymers, the J-aggregate absorption became dominant [16]. For fluorescence of the polymers in solution, J-aggregate fluorescence became prominent for the polymer having 33 PRU and was dominant for all higher-molecular-weight derivatives. The fluorescence of the polymer is subject to very strong “superquenching” by oppositely charged electron acceptors and energy-transfer quenchers. The degree of superquenching measured for these polymers equals or exceeds that observed for conjugated polyelectrolytes by the same kind of quenchers. Thus, it was found that Stern–Volmer

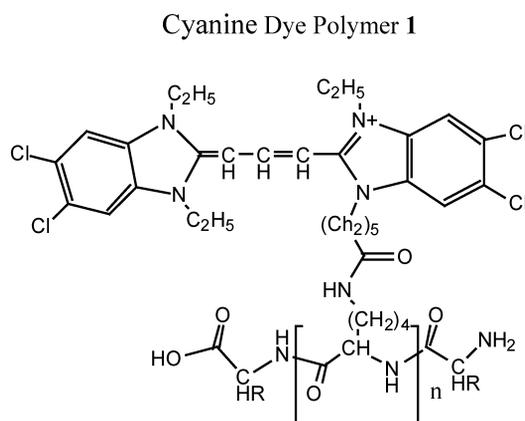


Fig. 1 Cyanine dye-pendant poly-L-lysine **1**. Synthesized in range of molecular weights, $n = 1$ –900. Synthesis: Ileana Place (UR), Roger Helgeson (UCLA).

quenching constants (K_{sv}) (which are not true constants but depend on polymer concentration) can reach as high as $1.2 \times 10^9 \text{ M}^{-1}$ for a polymer having ~ 900 PRU with **2** as the quencher (Fig. 2) [16]. The study of fluorescence quenching of a series of cyanine poly-L-lysine derivatives (**1**) with the an-

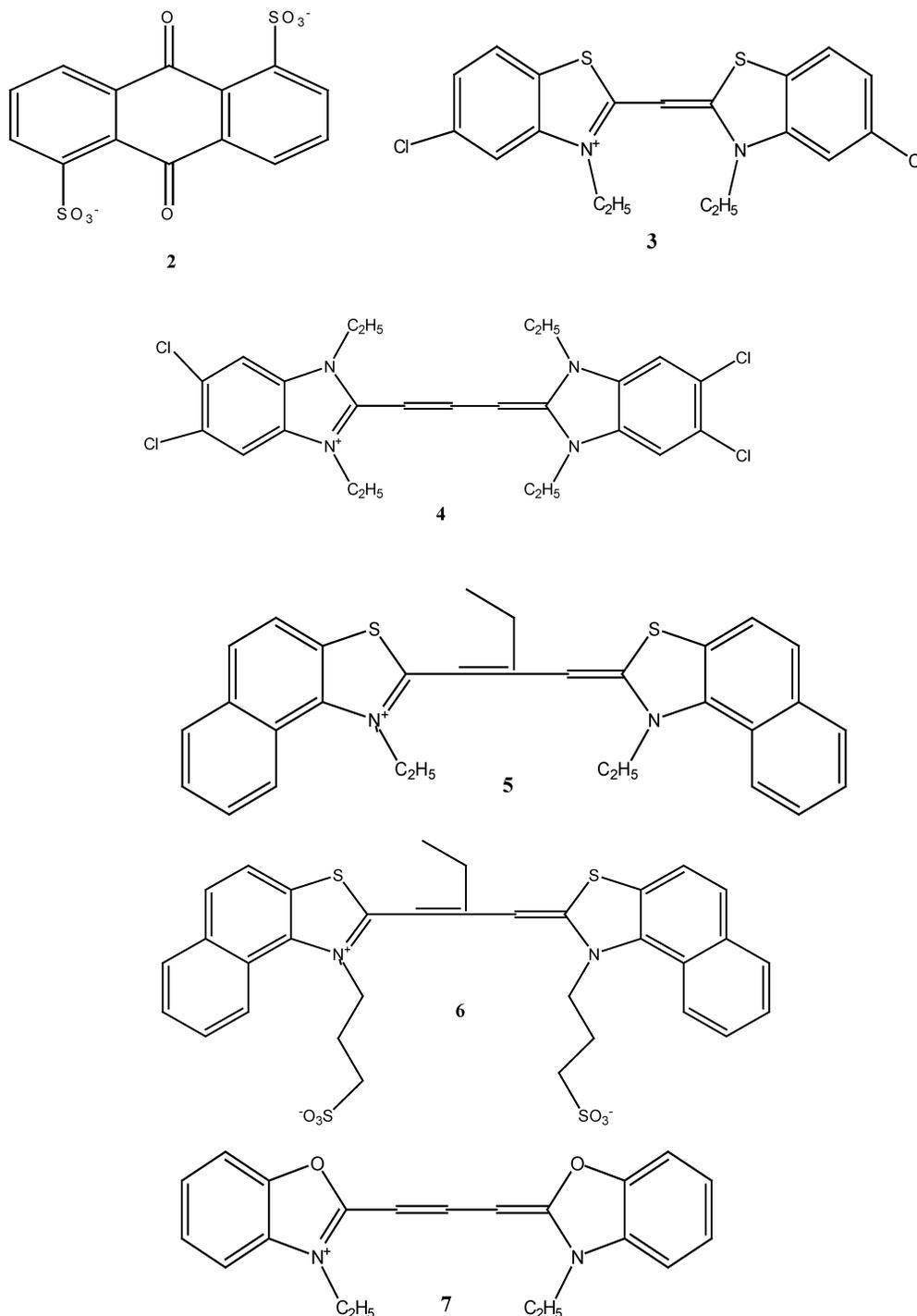


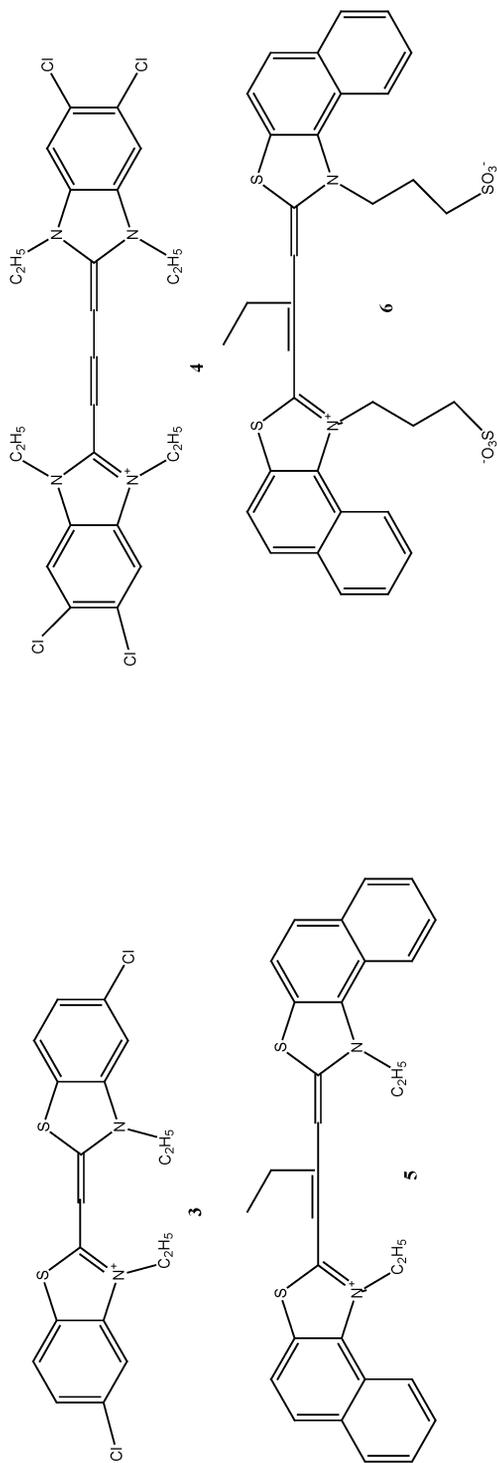
Fig. 2 Structures of cyanine dyes and quenchers used in this investigation.

ionic electron acceptor 9,10-anthraquinone-2,6-disulfonate (**2**) in aqueous solution showed that the K_{sv} values increase monotonically with polymer molecular weight; however, the interesting findings are that the value of PRU per quencher at 50 % quenching $(PRU/Q)_{50}$, which is a constant for any specific polymer-quencher combination, increases with the molecular weight of the polymer and then levels off at a maximum slightly greater than 100 for polymers having $N_{PRU} = 250\text{--}906$ [16]. The number of quenchers per polymer molecule at 50 % quenching reaches a minimum of ~ 2.6 for the polymer with 250 PRU [16]. When the same series of cyanine-poly-L-lysine having variable numbers of PRU is adsorbed onto silica microspheres or clay nanoparticles, both absorption and fluorescence spectra are predominantly J-aggregates regardless of the number of PRU [18]. For the polymer adsorbed onto silica, quenching of fluorescence by **2** is dramatically enhanced for the low-molecular-weight oligomers and even monomer, while the higher-molecular-weight polymers exhibit similar quenching to the same compounds in aqueous solution [18].

The “surface-mediated superquenching” observed for the monomer and low-molecular-weight oligomers has been found to be fairly general for a series of cyanine monomers assembling as J-aggregates by collection on Laponite clay nanoparticles [19]. As would be anticipated, quenching of cyanine ensembles by energy-transfer quenchers is generally more efficient (as measured by monomers/quencher at 50 % quenching $(M/Q)_{50}$ —analogous to $(PRU/Q)_{50}$ as defined above). The Laponite clay nanoparticles are relatively uniform in particle size; the particles are disk-shaped and thus the cyanine-coated regions consist of two domains on each face of the disk. It was estimated that the capacity for a single cyanine such as **3** or **4** should be ~ 1600 molecules per particle [19]. When single cyanines such as **3** or **4** adsorb onto Laponite they form J-aggregates which are somewhat different from J-aggregates of the same chromophores formed in different environments [19]. The fluorescence yields are low (0.02–0.08), and the absorption and fluorescence are broadened and somewhat blue-shifted compared to crystals or microcrystals [19]. The J-aggregate fluorescence of the clay-supported cyanines could be quenched either by incorporation of other cyanines or cationic quenchers into the adsorbed layer on the clay during its formation or by addition of oppositely charged quenchers from the aqueous solution in which the nanoparticles are suspended [16,17]. The behavior of assemblies of **3** and **4** with cyanine quenchers **5** and **6** is typical. **5** and **6** contain the same chromophore, a monomeric cyanine absorbing at $\lambda_{max} \sim 578$ nm. **5** is cationic and is added to the clay along with the predominant cyanine, while **6** is an anion, associating with the nanoparticles after J-aggregate formation. The J-aggregate of cyanine **3** on Laponite absorbs at 455 nm and emits at 470 nm (while an amphiphilic derivative with the same chromophore for which the alkyl chains appended to nitrogen are 18 carbons in length forms layers on silica microspheres absorbing at 466 nm and emitting at 476 nm). As shown in Table 1, the fluorescence of **3** is quenched by incorporating small amounts of **4** or **5** into the assemblies or by adding **6** to suspensions containing the precoated clay. All three cyanines quench the fluorescence of J-aggregates of **3** and the value of $(M/Q)_{50}$ is in the range 40–90, thus suggesting a modest degree of superquenching, modest perhaps due to the poor spectral overlap between the emission band of J-aggregated **3** and the absorption band of monomers **4**, **5**, and **6**. When similar experiments are carried out for the J-aggregate of **4**, it is found (as anticipated) that there is no quenching of the fluorescence of **4** by **3** but very strong quenching by **5** and **6**. The value of $(M/Q)_{50}$ is 430 for **5** and 200 for **6**; these correspond to 50 % quenching at levels of ~ 4 and 8 quenchers per nanoparticle. Interestingly, when mixtures of **3** and **4** are added to Laponite, the absorption spectrum that results corresponds roughly to the individual J-aggregates of **3** and **4** obtained when they are coated individually. The emission spectrum of the mixture corresponds to the J-aggregate of **4**, indicating that the lower energy J-aggregate efficiently quenches the higher energy aggregates. That the absorption spectrum of the dye mixture is not identical to the addition spectra of individual J-aggregates of **3** and **4** suggests that there must be a slight “mixing” of the different cyanines in the mixed aggregate assembly. Interestingly, when **3** and **4** are adsorbed individually on Laponite and the resulting suspensions mixed (1:1 molar ratio), the fluorescence from the individual cyanine J-aggregates rapidly evolves (tens of minutes) to a single weak emission corresponding to the J-aggregate of **4** [19]. This indicates the occurrence of a facile adsorption-des-

Table 1 Fluorescence quenching of cyanine aggregates on clay.

$$K_{SV}^{NP} = K_{SV} [S](A_s)$$



Quencher	K_S	K_S^N	$[S/Q]_S$	Quencher	K_S	K_S^N	$[S/Q]_S$
4	$4.7 \times 10^7 M^{-1}$	$23.5 \text{ nm}^2 \text{ molecule}^{-1}$	47	3	Negligible Quenching		
5	$8.9 \times 10^7 M^{-1}$	$44.5 \text{ nm}^2 \text{ molecule}^{-1}$	89	5	$4.3 \times 10^8 M^{-1}$	$215 \text{ nm}^2 \text{ molecule}^{-1}$	430
6	$4.0 \times 10^7 M^{-1}$	$20 \text{ nm}^2 \text{ molecule}^{-1}$	40	6	$2.0 \times 10^8 M^{-1}$	$100 \text{ nm}^2 \text{ molecule}^{-1}$	200

orption process for the individual cyanine molecules from the Laponite, even though essentially all of the cyanines are adsorbed on the nanoparticles. Not surprisingly, the “exchange” of cyanines between different nanoparticles may be eliminated by using water-insoluble cyanines in which the *N*-alkyl substituents are altered from 2- or 3-carbon chains to octadecyl derivatives.

Cyanines such as **3** and **4** also associate with water-soluble organic polyelectrolytes such as carboxymethylamylose (CMA) and carboxymethylcellulose (CMC). Addition of small amounts of CMA to aqueous or aqueous–methanol solutions of **3** results in the rapid formation of J-aggregate as evidenced by the characteristic absorption and fluorescence spectral changes over the range of CMA concentrations from 2.7 to 108 μM (Fig. 3) [20]. Although the kinetics of fluorescence emission from the aggregate has not yet been studied in detail, light emission was detected within 30 s of initiating the J-aggregation process, through the mixing of **3** and CMA. The emission maximum is at 470 nm, the same wavelength observed for fluorescence of **3** on Laponite in aqueous suspensions [19]; however, the fluorescence quantum efficiency of CMA is ~ 0.5 or almost five-fold higher than for the Laponite-adsorbed J-aggregate [20]. For addition of CMA to 20 % methanol + 80 % water solutions of **3**, there is a rapid conversion of monomer to J-aggregate which can be monitored by following fluorescence increases either with a spectrofluorimeter or multiwell plate reader. The J-aggregation is evidently controlled by the ratio of $(\text{CMA})_{\text{PRU}}/(\mathbf{3})$ and reaches a maximum in the range $(\text{CMA})_{\text{PRU}}/(\mathbf{3}) = 10\text{--}50$, depending on the degree of substitution (DS) of the CMA [20]. Figure 4 shows the linear increase in fluorescence intensity that occurs upon the addition of a solution of CMA (DS ~ 1.0) to **3** (concentration 10 μM); a plot of fluorescence intensity vs. $(\text{CMA})_{\text{PRU}}$ is linear ($r^2 = 0.957$) over the range of

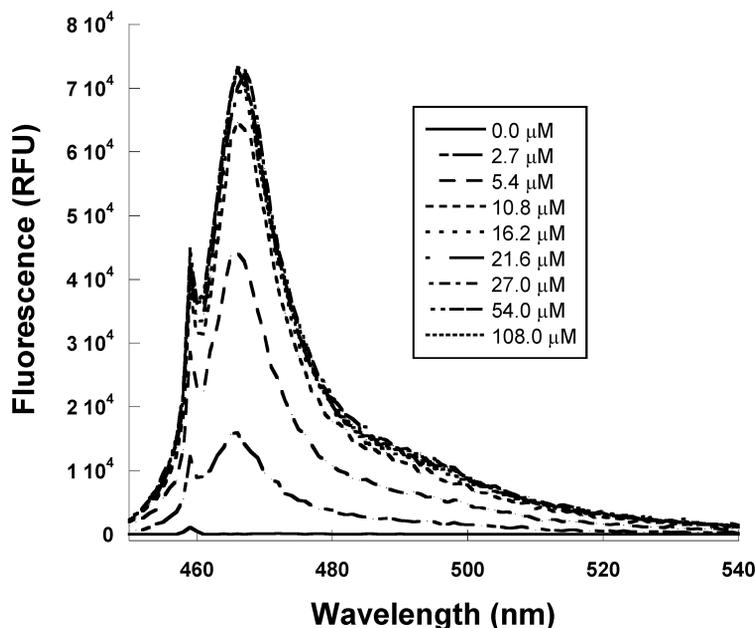


Fig. 3 Fluorescence emission spectra of cyanine (**3**) J-aggregate on CMA scaffold. Increasing volumes of an 8-mM stock solution of CMA in water were added to a 10- μM solution of cyanine (**3**) in 20 % methanol + 80 % water mixture. After each addition of CMA, the fluorescence emission spectra were captured after excitation at 460 nm. At first, the spectrum of cyanine (**3**) was taken before any CMA was added (lower-most tracing). The final concentrations of CMA (in μM) added were 2.7, 5.4, 10.8, 16.2, 21.6, 27.0, 54.0, and 108.0, respectively. The net increase in total reaction volume over the range of CMA titration was less than 3 %. The spectral changes are reflected by the increasing fluorescence intensity in the tracings. Fluorescence measurements were taken in a quartz cuvette using a SPEX fluorolog fluorimeter, and the data was analyzed using Datamax software package (Jobin Yvon Spex, New Jersey).

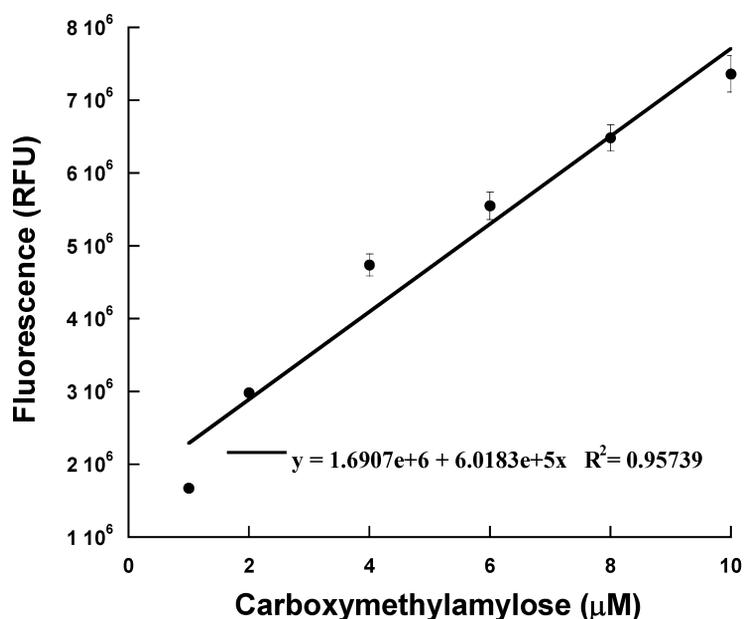


Fig. 4 Dose–response curve for CMA titrations. Cyanine (**3**) was dissolved to a final concentration of 10 μM in 20 % methanol + 80 % water mixture. Two hundred and fifty μL of this solution was dispensed into the wells of a 96-well white polystyrene microplate (OptiplatTM-96, PerkinElmer Life Sciences, Boston, MA). To the (**3**) solution was then added increasing and indicated concentrations of CMA, such that the net volume change in each well was 10 %. Fluorescence intensity from each cyanine (**3**)-CMA J-aggregate complex was then measured immediately. Fluorescence was measured using a Mithras LB 940 microplate spectrofluorometer (Berthold Instruments, Oak Ridge, TN). Samples were excited using a 355-nm excitation filter, and fluorescence emission was measured using a 460-nm emission filter; both filter sets were from Chroma Technology Corporation, Rockingham, VT. Values are average of triplicate measurements for each concentration of CMA added. Where not visible, error bars are masked within the symbol.

1–10 μM . For purposes of calculating, we used a figure of 242 as the molecular mass of CMA-PRU and 150 000 as the average molecular mass of amylose. Using a molecular mass of 242, we calculated the limit of detection (LOD) and limit of quantitation (LOQ) for CMA from **3** J-aggregate fluorescence to be 5 and 25 nM, respectively. When calculated using a molecular mass of 150 000 for the intact amylose molecule, the LOD and LOQ values are 8 and 40 pM, respectively. As higher concentrations of CMA are added, the J-aggregate absorption and emission level off and eventually decrease with increasing (CMA), evidently due to dissociation of the J-aggregate as extra CMA binding sites become available. A linear ($r^2 = 0.953$) dose–response was also observed when the CMA concentration was fixed at 40 μM and the concentration of **3** was varied between 1 and 10 μM (Fig. 5). The formation of J-aggregate for **3** upon addition of CMA is also accompanied by the development of a strong biphasic induced circular dichroism (CD) spectrum [20]. The CD spectrum changes from a positive to negative Cotton effect at the J-aggregate absorption maximum, indicating that the aggregate has a right-handed helical structure [20]. While CMA is a chiral polymer, it does not exist in a helical form in water due to electrostatic repulsion. The conformation for CMA in water is a heavily disrupted helix or random coil [21], depending on the DS of neutral glucose units with anionic carboxymethyl groups. The finding of a helical structure for the J-aggregate of **3** with CMA suggests that the helical aggregate formation is a cooperative self-assembly process [20]. The J-aggregate formed with CMA is remarkably stable, and a helical structure for the assembled **3**-CMA complex is indicated from studies of drop-cast solutions on hydroxylated silica by atomic force microscopy [20].

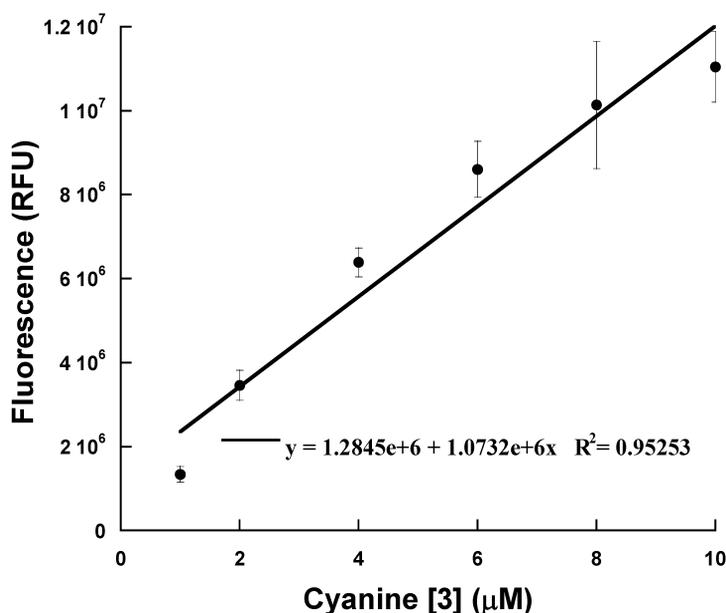


Fig. 5 Dose–response curve for cyanine (**3**) titrations. Increasing and indicated concentrations of cyanine (**3**) dye was dispensed into the wells of a 96-well microplate in a total volume of 250 μL . To the various concentrations of (**3**), CMA was added to a final concentration of 40 μM . All other conditions are similar to those described in the legend to Fig. 4.

A preliminary investigation of quenching of the fluorescence of the **3**-CMA complex has been carried out. In contrast to the behavior observed for J-aggregates of **3** and other cyanines on Laponite clay nanoparticles, neither **2** nor the cationic electron acceptor methyl viologen (MV^{2+}) produce significant quenching of the **3**-CMA complex. The failure of **2** to produce significant quenching may be attributed to repulsion from the anionic polymer. The reason why addition of the cationic electron acceptor MV^{2+} does not result in quenching of the J-aggregate is less certain; it may be that the J-aggregate of **3** is somewhat shielded within the complex and that the MV^{2+} cannot approach within a distance range close enough to allow electron-transfer quenching.

Interestingly, a cationic cyanine, **7**, which has good spectral overlap with the J-aggregate of **3** and thus should be a good energy-transfer quencher does exhibit moderate superquenching of the aggregate as shown in Fig. 6. As **7** is added to solutions containing the **3**-CMA J-aggregate, there is a quenching of aggregate fluorescence and a modest sensitization of the emission of **7**; the value of $(M/Q)_{50}$ is 33, somewhat lower than the values observed for quenching of the J-aggregates of **3** on Laponite.

The very strong fluorescence of the J-aggregate of **3** with CMA suggests that the formation or destruction of cyanine aggregates with anionic carbohydrate polymers might provide the basis for bioassays, especially for enzyme activity assays for those enzymes which destroy or modify carbohydrate polymers. We have recently found that similar formation of strongly fluorescent J-aggregates of **3** occurs with CMC and hyaluronic acid. For each polymer, there is a good range where J-aggregate fluorescence shows a linear increase with (PRU). In an attempt to develop a “scaffold disruption assay” we have focused on the enzymatic hydrolysis of CMA by amylase. The assay has been devised to operate as a fluorescence turn-off assay by monitoring enzymatic degradation of CMA in the range where the J-aggregate fluorescence is linear with $(\text{CMA})_{\text{PRU}}$.

Our initial assay development has been carried out with human saliva as the source of the enzyme amylase, in an “end-point” mode. Thus, CMA samples are incubated with the amylase for various time periods and aliquots from the incubated samples added to a solution of **3** in the wells of a 96-well micro-

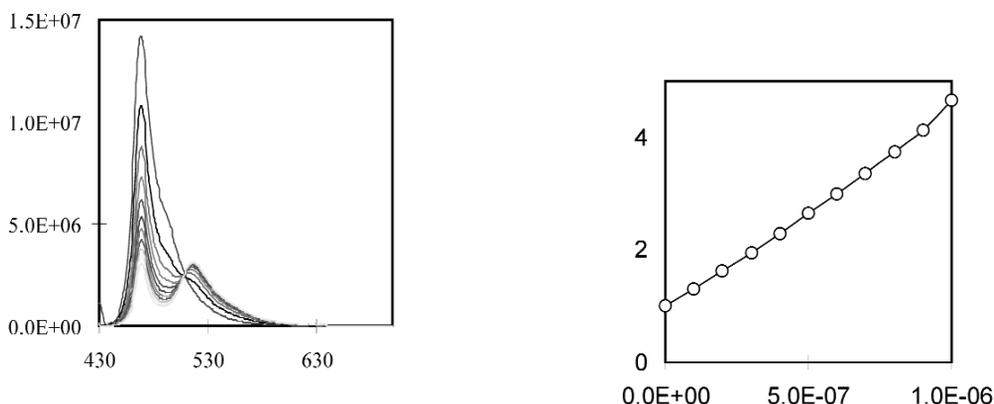


Fig. 6 J-Aggregate absorption and emission spectra and Stern–Volmer plot for fluorescence quenching. Details are presented in the figure itself.

plate. Preliminary results showed that the fluorescence of the 3-CMA J-aggregate decreases by increasing the incubation time of the amylase reaction. The chart (Fig. 7) shows the difference in the fluorescence intensity (Δ , fluorescence; relative fluorescence units, RFU) between reaction mixtures

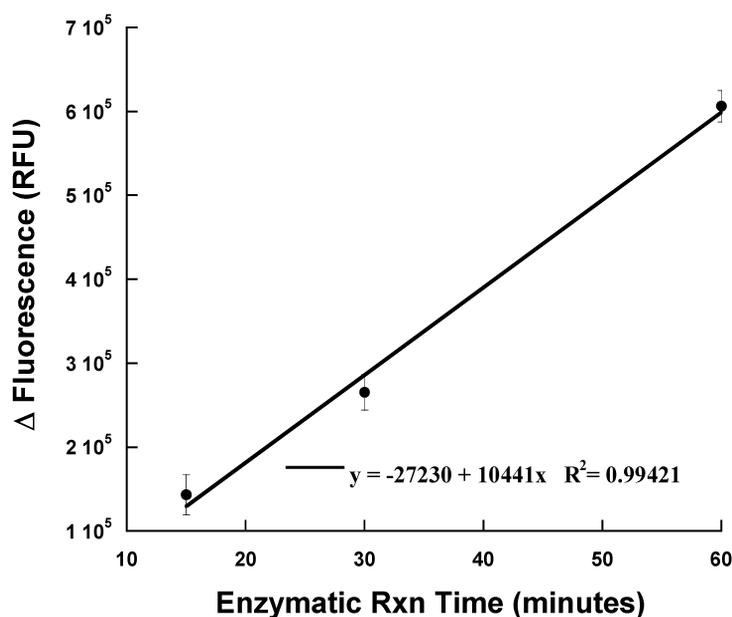


Fig. 7 Scaffold disruption assay–time course. Human saliva was collected from a volunteer and then centrifuged at 14 000 rpm ($16\,000 \times g$) for 1 min at ambient temperature ($\sim 25^\circ\text{C}$), using a Galaxy 16D microcentrifuge (VWR International, West Chester, PA). The resulting supernatant was centrifuged twice more as described above, collecting the supernatant following each centrifugation cycle. The supernatant from the third centrifugation cycle was used as a source of amylase enzyme activity in the “scaffold disruption assay.” CMA ($75\ \mu\text{M}$) was mixed with $5\ \mu\text{L}$ of saliva in a total volume of $60\ \mu\text{L}$ water and incubated at ambient temperature. At the indicated time intervals, one-half of the reaction volume was removed and added to $270\ \mu\text{L}$ of a $7.5\text{-}\mu\text{M}$ solution of cyanine (3), dispensed into the wells of a 96-well microplate. Fluorescence from the resulting J-aggregate was then immediately measured under conditions described in the legend to Fig. 4. Controls for these reactions were CMA mixed with saliva and added to cyanine (3) without any incubation period. Results are shown in the chart as the difference in fluorescence intensity (Δ , fluorescence) from reaction wells without and with incubation periods.

containing CMA and amylase without and with incubation time. A linear ($r^2 = 0.994$) increase in Δ RFU values was obtained with increasing reaction time (Fig. 7). Similarly, with increasing amylase amounts (increasing volumes of saliva), there was an increase in the Δ RFU, indicating enzyme-mediated destruction of the CMA scaffold (Fig. 8). The decrease in fluorescence of the 3-CMA J-aggregate (for a constant incubation time) also shows a dependence on the estimated amylase concentration as indicated in Fig. 8.

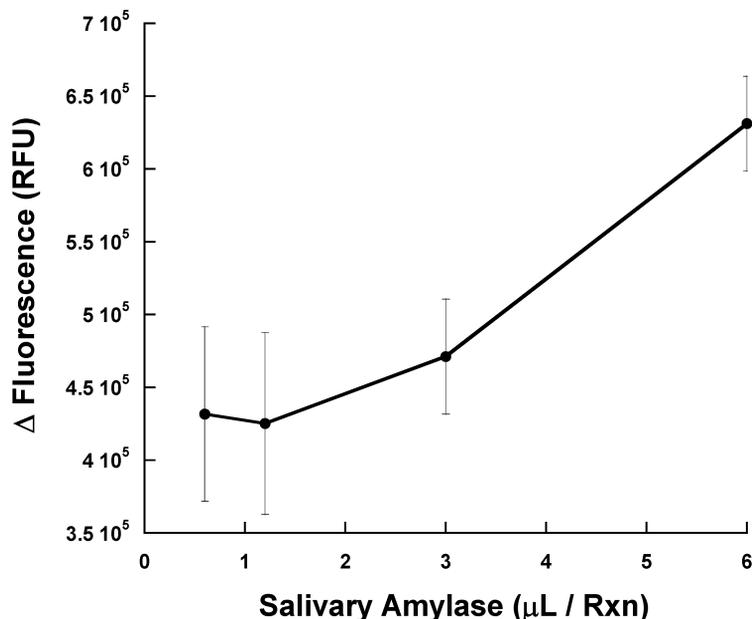


Fig. 8 Scaffold disruption assay–amylase concentration curve. Increasing and indicated volumes of saliva (amylase source) was mixed with CMA and incubated at ambient temperature for 60 min. All other assay conditions are identical to those described in the legend to Fig. 7.

While these experiments indicate there may be opportunities for development of “scaffold disruption assays” for a variety of biopolymers that exist either as helical structures on which J-aggregate formation is templated or for which cooperative self-assembly to form J-aggregates with cyanines can occur, there exists also the potential for interference by reagents which can disrupt the aggregation or which can themselves induce formation of J-aggregates. Since a “scaffold disruption assay” was demonstrated in a complex sample milieu such as human saliva (Figs. 7 and 8), the technology appears to have important implications for the sensing of a variety of biologically critical targets, both in the medical/health sciences as well as the biodefense arenas. An attractive possibility is the tuning of these assays by variation in the structure of the cyanine. Given the variety of cyanines that have been synthesized and the additional possibilities for modifying the structure while retaining a specific chromophore, it may be possible to develop assays that are both robust and specific for a given biopolymer.

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