Noncovalent Encapsulation Stabilities in Supramolecular Nanoassemblies

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Abstract: Exchange dynamics of lipophilic guest molecules, encapsulated in supramolecular nanoassemblies in aqueous solutions, have implications in evaluating the stability of drug delivery vehicles. This is because exchange dynamics is related to the propensity of a nanocarrier to be leaky. We describe a fluorescence resonance energy transfer (FRET) based method to evaluate guest exchange dynamics in the aqueous phase. We have utilized this method to analyze the stability of encapsulation in polymeric nanogels and other related amphiphilic nanoassemblies.

The complications faced in the administration of insoluble and toxic hydrophobic drugs to target sites have spurred tremendous interest in the field of delivery vehicle design.[1] Of the many factors considered in such design, the encapsulation stability of the delivery container is critical.[2] Encapsulation of a hydrophobic molecule in aqueous media itself is just an indicator of the thermodynamic distribution of the molecules between the container and the bulk solvent. This does not provide indications on the stability of encapsulation in terms of the dynamics of guest exchange with the bulk media. Understanding the dynamics of interchange between the bulk solvent and the nanocontainer is crucial, as this carries clear implications to the potential leakage of encapsulated guest molecules from the vehicles as they pass through a biological system. Thus, an analysis of this process is necessary for optimization of the design and construction of drug delivery carriers. To probe the stability of encapsulation, we describe here the dynamics of guest interchange in nanocarriers using Fluorescence Resonance Energy Transfer (FRET) as a tool. We then utilize this method to investigate the stability of encapsulation in a new class of polymer nanogels and compare it with that observed in classical amphiphilic nanoassemblies.

A lipophilic FRET pair, 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO, donor) and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, acceptor), was used for this purpose. These two dye molecules were independently sequestered in nanocarriers. When the solutions containing the dye molecules were mixed, two limiting scenarios are possible (Figure 1a). If the dye molecules are stably encapsulated and do not exchange the guest molecules with the bulk solvent environment, then the two dye molecules will continue to be in two separate nanocontainers. If this were the case, no FRET would be observed since the distance between the two dye molecules is much higher than their Förster radius. However, if there is a significant exchange of the guest molecules between the container interior and the bulk solvent when the two solutions are mixed, it is likely that the dye molecules will equilibrate between the two containers. This is because there would be no registry regarding the nanocontainer from which the dye molecule leaked. The resulting equilibration will cause DiI and DiO molecules to occupy the same container, leading to increased FRET.
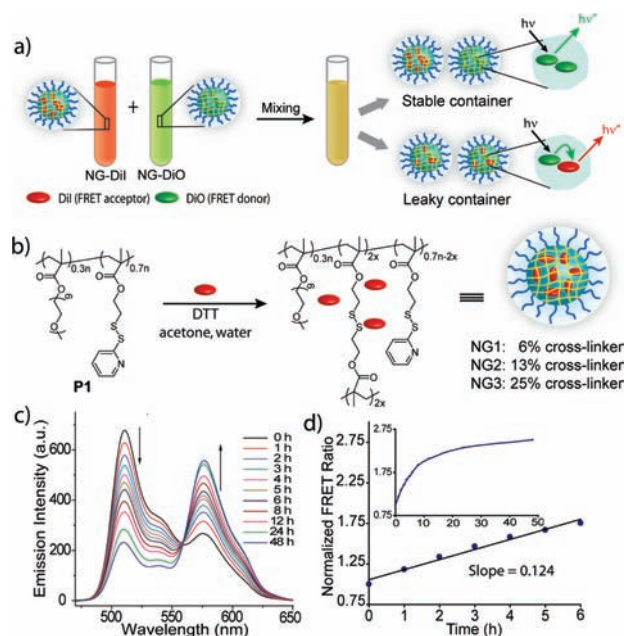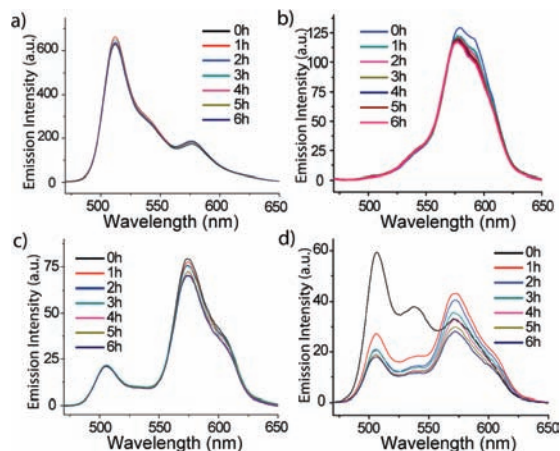

Figure 1. (a) Mixed nanogels encapsulated DiI/DiO and FRET behavior. (b) Synthesis of nanogels containing hydrophobic guest molecule. (c) Fluorescence emission spectra of mixed **NG1** encapsulated DiI/DiO. (d) Plot of FRET ratio vs time.

Thus, tracing the evolution of FRET in such systems provides insight into the dynamics of guest interchange and potential carrier leakage.

We sought to investigate the versatility of such an approach using cross-linked polymeric nanogels. The reason for this choice is the assertion that variations in cross-linking densities provide a method for tuning encapsulation stabilities and thus an opportunity to test our FRET-based strategy. Understandably, this feature also renders nanogels to be of great interest for controlled release applications in the area of pharmaceutical and biomedical research.[3] Generating water-soluble polymeric nanogels that encapsulate lipophilic molecules can be cumbersome. However, we have recently introduced a new emulsion-free method for nanogel preparation in aqueous solution that also provides for facile hydrophobic guest encapsulation (Figure 1b).[4]

To probe the guest exchange dynamics, aqueous solutions of **NG1** (6% cross-linked; ~200 nm in size) containing 1 wt % DiO or 1 wt % DiI were prepared by *in situ* loading (Figure 1b).[4,6] The two solutions containing the separate dyes, referred as NG1-DiO and NG1-DiI, were then mixed in water. Fluorescence from the DiO excitation (450 nm) was monitored over time. The evolution of FRET was obtained by tracing the decrease in the donor (DiO) emission and concurrent increase in the acceptor's emission (DiI). The results show that there is a gradual equilibration of the dye molecules over a 48 h period (Figure 1c). Interestingly, the thermodynamic distribution of the dye molecule significantly favors

Figure 2. FRET behavior of mixed nanocontainers encapsulated DiI/DiO: (a) **NG2**; (b) CTAB; (c) amphiphilic random copolymers; (d) pluronic block copolymer.

the nanogel interior, as discerned by the overall concentration of the otherwise insoluble dyes in the aqueous phase.[6] Thus, the continuous interchange of the dyes among the nanocarriers is responsible for observed enhancement in FRET. The FRET ratio $I_a/(I_d + I_a)$, where I_a and I_d are the fluorescence intensities of the acceptor (DiI) and the donor (DiO) respectively, was plotted against time (Figure 1d). The slope of the linear fit is related to the dynamics of the guest exchange, and we define this as the leakage coefficient (Λ), which was found to be $\sim$0.124 h^{-1} for the first 6 h in **NG1** (Figure 1d).

We envisaged that cross-linking density could be used to tune the rate of exchange/leakage. The preparation method indeed allows for control over the degree of cross-linking.[4,6] Accordingly, nanogels **NG1**, **NG2**, and **NG3**, with cross-linking densities of 6%, 13%, and 25% respectively, with encapsulated DiI and DiO were prepared. The dynamics of guest interchange were monitored, and the FRET ratios were plotted against time. **NG2** and **NG3** exhibited minimal exchange over 6 h at a Λ of 0.002 h^{-1} or below, compared to 0.124 h^{-1} for **NG1** (Figures 1c, d, and 2a).[6] These results suggest that the degree of cross-linking is effective in tuning guest exchange dynamics. Since the precursor polymer **P1** itself is capable of encapsulating DiO and DiI, we were interested in evaluating the exchange dynamics before and after cross-linking. **P1** exhibited a Λ of 0.739 after mixing, indicating rapid guest interchange as compared to the cross-linked nanogels.[6]

The key motivation in developing these experiments is to develop a screening method for probing exchange dynamics, and thus the potential, of drug delivery vehicles. Therefore, we were interested in investigating the guest exchange characteristics of other supramolecular assemblies that are known to encapsulate lipophilic guest molecules. The simplest supramolecular assembly that encapsulates lipophilic guest molecules involves small molecule surfactant-based micelles. Accordingly, we encapsulated DiO and DiI in CTAB (cetyl trimethylammonium bromide) and Tween80 micelles and carried out the FRET experiments. Complete equilibration of the dye molecules was observed instantaneously upon mixing the DiO and DiI based micelles with the Λ of 0.850 and 0.501 respectively (Figure 2b).[6] Since the nanogels are based on polymers, we also investigated other polymer-based nanoassemblies. We measured the guest exchange dynamics of an amphiphilic random copolymer (structure shown in Supporting Information).[7] The Λ in these assemblies was found to be 0.788 (Figure 2c). Similarly, the encapsulation stability of pluronic block copolymer micellar assemblies,[8] which are widely used in biomedical applica-
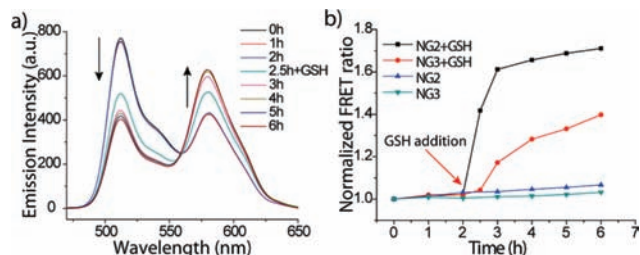
Figure 3. Release of encapsulated DiI/DiO: (a) **NG2** added GSH at 2.5 h; (b) dynamics of interchange of **NG2** and **NG3** with/without adding GSH.

tions, exhibited a Λ of 0.358 (Figure 2d). This is slower than assemblies based on small molecule surfactants and random copolymers but faster than the nanogels. It is also interesting to note that the assemblies based on the pluronic copolymers exhibit some inherent loss of sequestered dye molecules, as seen from the loss of emission intensity from both dyes over time (Figures 2d and S5).

The results above suggest that the cross-linked polymer nanogels exhibit high encapsulation stability and the leakage dynamics can be tuned by varying the cross-linking density. While encapsulation stability is important, a practical delivery vehicle must also be able to release its contents in response to a biologically relevant stimulus. As the nanogels consist of biodegradable disulfide cross-linkers, the release of encapsulated molecules can be potentially triggered upon exposure to reducing agents. To test this, we utilized **NG2** that exhibited very stable encapsulation. Glutathione (GSH, 10 mM), a disulfide reducing agent present at higher concentrations inside cells, was added to the solution containing NG2-DiO and NG2-DiI after 2.5 h. Significant increase of the FRET ratio was observed at this point (Figure 3a and 3b). This is presumably caused by dye leakage upon cleavage of the disulfide cross-linkers due to the presence of GSH, which loosens the gel and reduces encapsulation stability. Moreover, significant decreases in the emission intensities of the individual dyes indicate that the GSH reaction reduces the distribution coefficient of these dyes as well.[6] These results, combined with experiments with **NG3**,[6] confirm that the dynamics of guest interchange can be controlled by cross-linking density and that release can be externally triggered (Figure 3b).

In summary, we report on a FRET-based method to monitor the guest exchange dynamics in water-soluble nanoassemblies, which provides insight into the leakage characteristics of these nanocontainers. We have thus defined a leakage coefficient parameter, Λ, from the FRET experiments and analyzed this value for a variety of nanoassemblies. We find that the guest exchange is slower in cross-linked polymer nanogels and that this can be conveniently tuned by altering the degree of cross-linking. We have also investigated the relative guest exchange rates in other amphiphilic nanoassemblies. We find that while pluronic block copolymers exhibit higher encapsulation stabilities compared to small molecules and random copolymers, they do not compare to those offered by cross-linked polymer nanogels. In addition, we show that the stably encapsulated guests in the nanogels can be released in response to an external trigger. The dyes employed model the hydrophobic nature of drug molecules that commonly face solubility issues during administration. Thus, understanding the dynamics of encapsulated guest exchange between nanocarriers, using this method, provides a useful starting point for evaluating viable drug delivery vehicles. Moreover, the exchange dynamics provide information on the possibility of utilizing a vehicle to deliver multiple drugs in separate containers with timed release. These FRET-based studies

also highlight the advantages of the newly developed polymeric nanogels in drug delivery applications.

Supporting Information Available: Experimental details and fluorescence data. This material is available free of charge via the Internet at http://pubs.acs.org.

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