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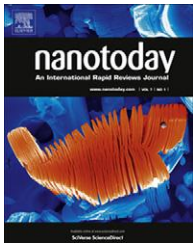

REVIEW

Polymeric micelle stability

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Summary Polymeric micelles provide a platform that can be carefully tuned for drug delivery. The nano-scale aggregates form spontaneously in aqueous solution and can be used to overcome drug insolubility and increase circulation half life. Self-assembled polymeric micelles are dynamic in nature; thermodynamics defines how the system acts as micelles approach equilibrium, while kinetics characterizes the system's behavior over time. In this review, we discuss factors that affect the stability of self-assembled polymeric micelle systems for drug delivery and methods used to study stability. Considerations of polymer composition, drug encapsulation, and environmental conditions influence polymeric micelle stability. Ultimately, we emphasize the importance of investigating micelle systems in physiologically relevant media to improve therapeutic efficacy and reduce systemic toxicity in clinical applications.

Introduction

Advances in drug discovery due to combinational chemistry and high throughput screening have greatly increased the number of potential therapeutic drugs [1]. However, almost one-third of the compounds are poorly soluble in water and cause systemic toxicity [2,3]. Therapeutic drugs are transported through the bloodstream, therefore, solubility directly affects absorption and physiological distribution [4]. Copolymers that self-assemble into micelles are being investigated as vehicles to increase the solubility and decrease the toxicity of hydrophobic drugs. Among these, a unique class of amphiphilic polymers aggregate to form nano-scale carriers (~10—200 nm) that can encapsulate hydrophobic drugs in the core and allow for biocompatible modifications at the surface.

The paradigm of utilizing polymeric micelles to encapsulate therapeutic agents emerged nearly 30 years ago from the labs of Ringsdorf et al. [5—12]. Although micelle formation from block copolymers was well-known at that time [13—15], the majority of micelles were not synthesized from biocompatible materials. For therapeutic delivery, a new class of amphiphilic carriers was required. These were designed to mimic natural carrier systems, such as viruses, and intentionally composed of relatively bio-inert materials. The amphiphilic copolymers self-assembled into micelles in aqueous environments with the hydrophobic polymer forming the core of the micelle and the hydrophilic polymer forming the corona (Fig. 1). Small molecule hydrophobic

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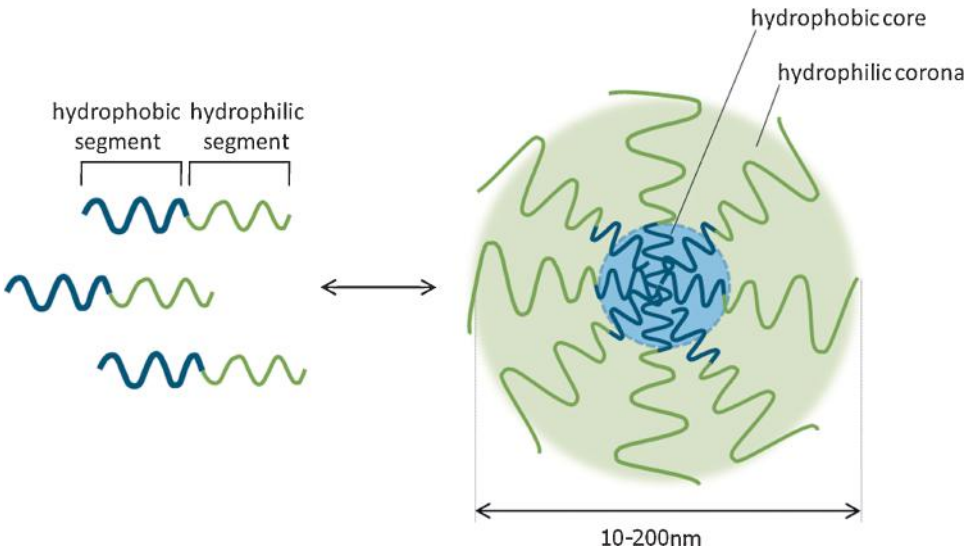

Figure 1 Schematic illustration of polymer micelle formation. Amphiphilic copolymers self-assemble to have a hydrophobic core and a hydrophilic corona structure in aqueous environments.

drugs were soluble in the hydrophobic core and thereby encapsulated within these amphiphilic polymeric micelles.

Self-aggregating, biocompatible, polymeric micelles (hereafter referred to as ''micelles'') are promising, yet the micelle must meet stringent design criteria for success. The micelle must: (i) be small enough (~10—200 nm) to effectively penetrate into tissue; (ii) be unrecognizable by the mononuclear phagocyte system (MPS) for a sufficient time to allow accumulation in target tissue; (iii) be eliminated from the organism either after degradation or dissolution; (iv) locate and interact with the target cells; (v) have tunable stability; (vi) improve the pharmacokinetic (PK) profile of the encapsulated drug cargo; (vii) possess high loading capacity; and (viii) be synthesized in a reproducible, facile method which is reasonably inexpensive [6,8,16—18].

Despite this daunting list of expectations, there has been increasing interest and research into micelles since their introduction and several systems are now being evaluated clinically [19—22]. Polymeric micelles have found particular utility in the delivery of cancer therapeutics based on physiological changes associated with the tumor, such as over-expression of cell surface receptors in cancerous tissue and the enhanced permeability and retention theory [23,24]. Currently, six micelle drug delivery formulations for cancer treatment are in clinical trials. Representative PK data from two of these systems, NK911 and SP1049C, in phase 1—3 clinical trials show 3.5-fold (NK911) [19] and 3.1-fold (SP1049C) [22] increase in the circulation time of micelle formulations of doxorubicin compared to the free drug. For both micelle systems, the elimination half life ($t_{1/2,\beta}$) (2.8 ± 0.3 h for NK911 and 2.4 ± 2.1 h for SP1049C) value was improved over that for the free drug (1.4 ± 0.6 h). Disappointingly, systemic toxicity matched closely with free drug formulations. As such, the pioneering micelle formulations have seen only limited clinical success.

Several characteristics limit the efficiency of modern micelle systems including inadequate drug loading capacity, poor stability in blood, and insufficient binding and uptake by cells. These limitations have been generally discussed in recent review articles [18,25,26]. Among confounding issues, the inherent instability of micelles remains a significant challenge. Improving the stability of micelles under physiological conditions may lead to dramatic improvements in PK and thereby unlock the door to successful clinical applications of micelle systems.

The goals of this review are to inform the reader of factors that influence the stability of self-assembled polymeric micelle systems used for drug delivery, to discuss current methods being utilized to study stability, and to emphasize the importance of investigating micelle systems in physiologically relevant media to improve clinical translation.

Structure and properties of micelles

The most common polymeric micelles used in drug delivery are amphiphilic di-block (hydrophilic—hydrophobic) or tri-block (hydrophilic—hydrophobic—hydrophilic) polymers. Additional structures include graft (hydrophilic-*g*-hydrophobic) or ionic (hydrophilic-ionic) copolymers. In almost all systems, the hydrophilic segment is composed of poly(ethylene glycol) (PEG) [16,27,28]. While alternative hydrophilic polymers such as poly(ethylenimine) [29,30], poly(aspartic acid) [31], poly(acrylic acid) [32], and dextran [33] have been studied, most novelty in micelle composition is derived from the choice of hydrophobic or ionic segments.

The defining characteristic of micelle systems is the ability of polymer units to self-assemble into nano-scale aggregates. Self-assembly is a thermodynamic process. The potential for self-assembly is determined by the mass and composition of the copolymer backbone, the concentration of polymer chains, and the properties of encapsulated or pendant drugs and targeting agents. The contributions of each of these factors are discussed in detail below.

Critical micelle concentration (CMC)

Amphiphilic polymers self-assemble in aqueous solutions with the hydrophobic chains aggregating together to form

the core and the hydrophilic chains extended towards the aqueous environment. In this way, the hydrophilic chains shield the hydrophobic chains from interaction with water, reducing the interfacial free energy of the polymer—water system. Minimizing interfacial free energy is the main driving force for micelle formation. In aqueous solutions, the hydrophobic effect is the major mechanism for decreasing interfacial free energy [34,35].

The critical micelle concentration (CMC) is the minimum concentration of polymer required for micelles to form. At low polymer concentrations, there are insufficient numbers of chains to self-assemble and instead the chains are found distributed throughout the solution and act as surfactants, adsorbing at the air—water or aqueous—organic solvent interface. As the concentration of polymer increases, more chains are adsorbed at the interface. Eventually a concentration is reached at which both the bulk solution and interface are saturated with polymer chains — this is the CMC. Adding more polymer chains to the system beyond this point will result in micelle formation in the bulk solution to reduce the free energy of the system. At high polymer concentration, the micelles are stable unless they are diluted below the CMC. The micelles will then disassemble and free chains are again found in the bulk solution and adsorbed at the air—water interface or aqueous—organic solvent interface.

While micelles are often pictured as spheres, it is critical to recognize that the micelles are not always spherical and not solid particles. The individual polymer chains that form a micelle are in dynamic equilibrium with chains that remain in the bulk solution, at the solvent interface, and incorporated into adjacent micelles.

Size and aggregation number

The number of polymer chains that assemble to form a micelle is the aggregation number [36,37]. In its simplest form, the aggregation number (N_{ag}) is given by the equation:

$$N_{ag} = \frac{M}{M_0}$$

M is the molecular weight of one micelle and M_0 is the molecular weight of the polymer backbone. Direct determination of the molecular weight of a micelle using centrifugal sedimentation or other techniques can be difficult; however, estimates of M can be found by calculating the size of the micelle, as in the equation:

$$M = \frac{4\pi N_A R^3}{3\nu^2}$$

Here, R is the radius of the micelle, N_A is Avogadro's number, and ν^2 is the partial specific volume of the polymer. Using a second approach, N_{ag} can be calculated by determining the hydrodynamic radius of a micelle and measuring intrinsic viscosity, as in the equation:

$$N_{ag} = \frac{10\pi R_H^3 N_A}{3[\eta]M}$$

where R_H is the hydrodynamic radius and $[\eta]$ is the intrinsic viscosity. The aggregation number for micelles usually varies from tens to hundreds, but also has been reported in the thousands [38—40]. The majority of polymeric micelles are spherical and fall in the size range of 10—200 nm [16—18,41—44]. The structure, molecular weights and molar mass ratio between hydrophilic and hydrophobic segments of the polymer backbone have a direct impact on the size and shape of assembled micelles (see 'Contributions to stability from hydrophobic segments' section). In general, when the hydrophilic polymer segment (corona) is longer than the hydrophobic polymer segment (core), spherical micelles are favored; however, an increase in the number of crystalline folds in the core leads to a reduction in corona crowding, favoring rod-like morphologies (see 'Contributions to stability from hydrophobic segments' and 'Impact of encapsulated and conjugated drug' sections). As micelles are physically assembled structures, environmental changes often result in size changes and therefore impact micelle stability. In addition, micelle size is determined by the molecular geometry of the individual chains which are influenced by solution conditions such as ionic strength, pH, temperature, and polymer concentration ('Environmental influence on micellization' section).

Stability

Micelles undergo a number of environmental changes upon intravenous injection, including significant dilution, exposure to pH and salt changes, and contact with numerous proteins and cells. For use as drug delivery vehicles, micelles must remain intact to prevent drug cargo release before reaching the target cells. For use in drug solubilization or local drug delivery, micelles must remain intact during formulation and administration. The stability of micelles can be thought of generally in terms of thermodynamic and kinetic stability. Thermodynamic stability describes how the system acts as micelles are formed and reach equilibrium. Kinetic stability describes the behavior of the system over time and details the rate of polymer exchange and micelle disassembly.

Thermodynamic stability

The CMC is a fundamental parameter used to characterize the thermodynamic stability of micelles. It is related to thermal energy, k_BT, and the effective interaction energy between polymers and the bulk solution, ε_h, in the equation, $\mathrm{CMC} = \exp(-n\varepsilon_h / k_BT)$. Lower values indicate greater thermodynamic stability. CMC is also directly related to the standard free energy of micellization, ΔG°_{mic}, in the equation, $\Delta G^{\circ}_{mic} = RT\ \ln(\mathrm{CMC})$ [45]. Polymeric micelles exhibit lower CMC values than low molar mass surfactant micelles because the polymer chains have many more points of interaction than small molecules. Polymer solutions exhibit different physical properties below and above the CMC. Typically, polymeric micelle CMCs are at micromolar concentrations [46,47]. The length of the hydrophobic segment correlates directly with stability [48,49]. The propensity for micelles to dissociate is related to the composition and cohesion of the hydrophobic core [50]. Increasing the

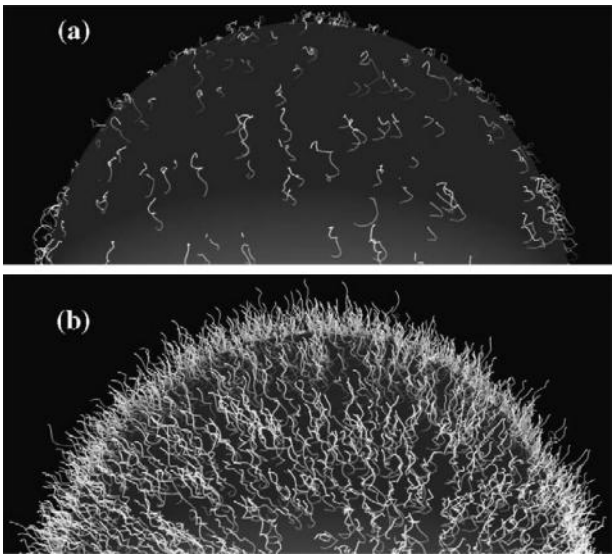

Figure 2 Schematic diagrams of PEG configurations on the upper hemisphere of a polymeric nanoparticle. In (a), the low surface coverage of PEG chains leads to the ''mushroom'' configuration where most of the chains are located closer to the particles surface. In (b), the high surface coverage and lack of mobility of the PEG chains leads to the ''brush'' configuration where most of the chains are extended away from the surface. Reprinted from International Journal of Pharmaceutics 307, Peppas, NA, et al., Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles, 93—102. Copyright 2006 with permission from Elsevier.

hydrophobicity of the copolymer increases the cohesion of the hydrophobic core and results in a lower CMC [48,51]. For micelles used in drug delivery, the drug—core interaction can also affect stability. An encapsulated, hydrophobic drug may stabilize the micelle through additional hydrophobic interactions between the core and the drug [52].

Thermodynamic stability is also influenced by the interactions between polymer chains in the corona with each other and with the aqueous environment. Most micelles for drug delivery employ PEG as the hydrophilic segment. Individual PEG chains interact by intermolecular van der Waals forces; the PEG chains interact with water in the bulk solution by hydrophilic interactions, such as hydrogen bonding/dipole—dipole forces [27]. Increasing the PEG chain length and surface density will force the polymers to adopt more rigid and extended, brush-like conformations (Fig. 2). In contrast, low MW PEG and low surface density of PEG results in limited surface coverage of the micelle, leading to aqueous exposure to the hydrophobic core and micelle destabilization. Sufficient hydrophilic polymer surface coverage is required to allow fluid movement of surface chains while also preventing exposure of the hydrophobic core ('Destabilization from environmental factors' section for details on destabilization mechanisms).

Measuring thermodynamic stability

The CMC can be determined by measuring sharp changes in physical parameters that occur at the CMC, such as micelle size, optical clarity of a solution, surface tension measurements, and viscosity. The most common methods, which are chosen due to high sensitivity, include HPLC, particle size by light scattering, and fluorescence spectroscopy [28].

HPLC-based gel permeation chromatography is used to separate single polymer chains from those aggregated into micelles. The main method of detection is UV—vis, normally set to detect absorbance at low wavelengths of approximately 220—280 nm. The absorbance can be quantified by vigilant users from a set of standards to estimate aggregation number and CMC. As detection is non-specific, there is the potential spectral overlap of polymer chains that makeup the micelle and any encapsulated/conjugated molecules. In addition, the relatively large size of micelles often results in similar retention times between micelles and components of the solution. This is especially true in more complex media containing proteins.

Static light scattering (SLS) and dynamic light scattering (DLS) techniques provide a rapid, high-throughput means to determine CMC, micelle size and polydispersity of the sample [53]. In DLS measurements, when light hits the micelles suspended in solution, it scatters in all directions and the scattering intensity, in relation to time, is used to determine the size and distribution of particles in solution. Current light scattering techniques are limited to polymer concentrations above 50 ppm and are limited in resolving the contribution from a single population in media with multiple components.

To determine the CMC using fluorescence techniques, fluorogenic dyes are encapsulated in assembled micelles and the release of the dye is measured using a spectroflourometer at varying micelle concentrations [48]. The hydrophobic fluorogen, pyrene, is a popular agent due to its tendency to associate within the micelle core and because it can be used to determine CMC onset at polymer concentrations lower than other techniques ($\sim$1 ppm for PEO-*b*-PS). When pyrene is added to polymer solutions, it partitions into the hydrophobic core during micellization and its spectroscopic properties change [28]. There are three changes that can be observed based on pyrene's characteristic behavior in aqueous *versus* hydrophobic environments: (i) a red shift in the excitation spectrum maximum from 332 nm to 338 nm; (ii) a decrease in the intensity ratio of the first and third vibrational bands of pyrene (I_1/I_3); (iii) an increase in the fluorescence lifetime from $\sim$200 ns to $\sim$350 ns [54]. Fluorescence experiments should be closely monitored because emission is also sensitive to environmental factors including temperature, pH, and ionic strength.

The CMC in solution can also be determined by measuring surface tension. A Wilhelmy plate or a Du Noüy ring is used to measure surface tension of solutions with various concentrations of polymer. The CMC is indicated by a sharp decrease in the surface tension as a function of concentration and signifies the saturation of polymer at the air—water or aqueous—organic interface [55]. Measuring the surface tension arguably provides the most direct determination of micelle formation, but these methods can be technically challenging. Separate investigations by Moran et al. [56] and Erbil [57] show that the speed of the ring or plate being removed from solution can change the surface tension measurement.

Contributions to stability from hydrophobic segments

For block copolymer micelles, increased hydrophobic chain length correlates with increased stability (and therefore reduced CMC). Gaucher et al. demonstrated that increasing the percent of poly(D,L-lactide) composition in a polyvinylpyrrolidone-*block*-poly(D,L-lactide)-*block*-polyvinylpyrrolidone (PVP-*b*-PDLLA-*b*-PVP) tri-block copolymer decreased the CMC value [50]. Similarly, a study in Kwon's lab showed that increasing the hydrophobic acyl chain length decreased the CMC in poly(ethylene oxide)-*block*-poly(N-hexyl-L-aspartamide)-acyl copolymers [49,58].

Importantly, there must be a balance between the hydrophobic and hydrophilic chain lengths on the copolymer for maximum stability. Beyond a certain point, increasing the hydrophobic chain length leads to micelles of less uniform shape, resulting in non-spherical aggregates. Notably, Winnik and co-workers have generated complex micelle architectures from di-block copolymers by controlling the hydrophobic to hydrophilic block ratio [59].

In addition to the length of the hydrophobic block, the hydrophobicity of the core influences micelle stability. Measurements of the CMC for PEG-*block*-poly(alkylmethacrylates) of differing degrees of hydrophobicity demonstrated that the most hydrophobic copolymer had the lowest CMC as determined by steady state pyrene fluorescence [51]. Furthermore, Van Domeselaar et al. completed light scattering measurements on PEO-*b*-peptides with various peptide sequences. Polymers synthesized from more aliphatic and aromatic peptide cores had lower CMC values [48]. Increased hydrophobicity and stacking interactions in the core decreased the CMC. In contrast, micelles formed from mixtures of two hydrophobic copolymer chains had much higher CMCs. The authors suggested that hydrophobicity alone is insufficient to predict stability, and that intermolecular interactions in the micelle core, such as stacking interactions, resulted in a ''glassy'' state in the core that influences stability. Mixed peptide cores had more disordered secondary structures than homopolymers resulting in greater exposure of the polar chains to the surrounding medium, preventing micellization.

The above examples demonstrate that the composition of the hydrophobic chain is paramount in micelle design. In general, increasing the hydrophobic chain length and degree of hydrophobicity leads to more stable micelles; however, there is little detail into how far these parameters can be increased before their respective contributions start to plateau. Additionally, examples in the literature investigating mixed micelles are limited making it difficult to decipher if the stability of micelles is the result of generic ''hydrophobicity'' or specific to the interactions between copolymer chains.

Impact of encapsulated and conjugated drug

Drug—core interactions also influence micelle stability. Lee et al. investigated the CMC of PEO-*b*-PLA micelles with varying amounts of carboxylic acid in the hydrophobic segment [52]. The release of the encapsulated drug, papaverine, was also monitored in terms of cohesive forces between drug and polymer core. There was an increase in drug loading efficiency when the block copolymer contained more carboxylic acid functionality for ion interactions with papaverine. This enhanced interaction between drug and micelle core lowered the CMC and also decreased the release rate of the drug.

Allen's lab investigated the differences in drug stability and morphology between micelles loaded with docetaxel and micelles that were conjugated with docetaxel and then loaded with additional drug. Docetaxel was conjugated to the hydrophobic portion of poly(ethylene glycol)-*block*-poly(ε-caprolactone) (PEG-*b*-PCL) micelles [60]. Docetaxel was then encapsulated into both PEG-*b*-PCL and PEG-*b*-PCL-docetaxel micelles. Conjugated micelles showed an 1840-fold increase in aqueous solubility of the drug and more uniform, spheroid morphology (Fig. 3). The favorable increase in drug solubilization and more uniform shape are attributed to the increased hydrophobic interactions between conjugated and encapsulated drug in the micelle core. Such a dramatic increase in solubility is encouraging. It is neither clear whether these characteristics are consistent with other conjugated/encapsulated drug micelles nor whether a similar effect could be achieved with another small hydrophobic molecule.

Environmental influence on micellization

The microenvironment is a major factor for micelle formation and stability. Two common methods for micellization are dialysis and co-solvent evaporation [61]. In the former method, polymer is dissolved in organic solvent which is then removed by dialysis against an aqueous buffer. In the latter method, polymer is dissolved in mixtures of organic and aqueous solvents and the organic solvent is then removed by spray drying or rotoevaporation. To demonstrate the reliance of micellization on preparation method, Meli et al. showed that micelles prepared from two methods had significantly different sized particles and distributions, as determined by dynamic laser light scattering [62]. Here, micelles prepared from co-solvent evaporation were smaller ($\sim$30 nm) and more uniform with a polydispersity index (PI) of 0.07 while dialysis produced much larger ($\sim$110 nm) and more disperse micelles with a PI of 0.27. The authors suggest that the difference between micelle size is path-dependent and may be a result of changes in rates to reach equilibrium. The disparity between micelle size based on preparation methods is intriguing. Okano and co-workers have suggested that even small changes (*e.g.* temperature and molecular weight cut-off of dialysis membrane) in a single preparation method can have a dramatic impact on micelle size [63].

Changes in solvent conditions have a dramatic effect on the CMC and size of micelles formed as well [62,64—67]. Cheng et al. tested assembly of PLGA-PEG micelles in different solvent formulations by altering the type and concentration of organic solvent used to solubilize the polymer chain for a given solvent/water system [68]. Four common solvents with varying degrees of water miscibility — THF, DMF, acetone, and acetonitrile — were investigated. Results show a general correlation between solvent/water miscibility and micelle size where an increase in miscibility led to a decrease in micelle size. Similar results from Lavasanifar's lab demonstrate the impact of solvent selection on size and dispersity of micelles formed from methoxy poly(ethylene oxide)-*block*-poly(ε-caprolactone) (MePEO-*b*-PCL) [67]. The use of acetone as the organic solvent produced micelles with an average diameter of 87.8 ± 9.4 nm and a relatively

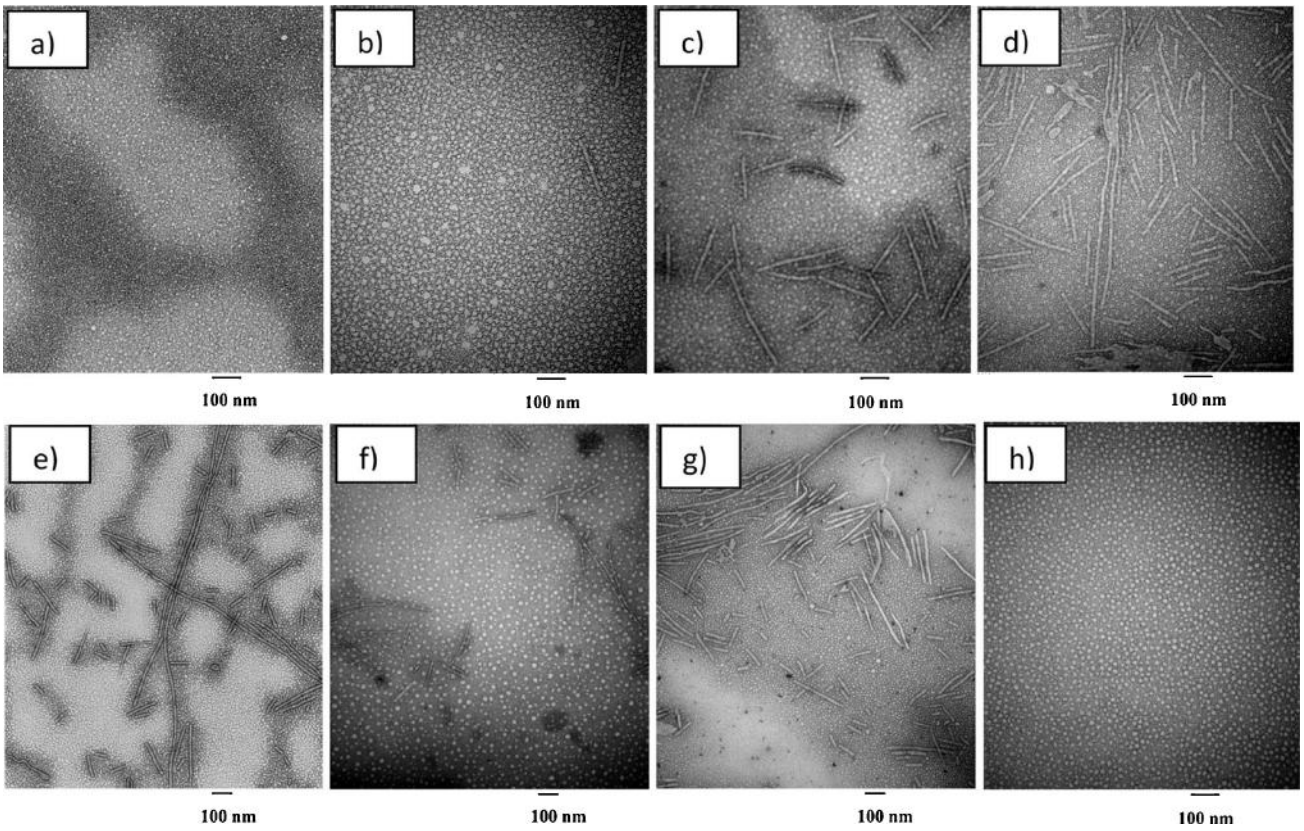

Figure 3 TEM images of micelles formed from mixtures of PEG-*b*-PCL(2k1k)-DTX and PEG-*b*-PCL(2k1k) in molar ratios of (a) 9:1, (b) 7:3, (c) 3:7, and (d) 1:9. Micelles containing physically entrapped DTX (2% w/w): (e) PEG-*b*-PCL (2k2k), (f) PEG-*b*-PCL(2k1k) + DTX, (g) PEG-*b*-PCL(2k2k) + DTX, and (h) PEG-*b*-PCL(2k1k)-DTX + DTX. The concentration of all copolymers and the copolymer-drug conjugate was 10 mg/mL. In brush micelles where the hydrophilic polymer segment (corona) is longer than the hydrophobic polymer segment (core), spherical micelles are generally favored; however, an increase in the number of crystalline folds in the core leads to a reduction in corona crowding, favoring rod-like morphologies. In this example, the morphology of PEG-*b*-PCL(2k1k) and PEG-*b*-PCL(2k2k) copolymer micelles show a mixed population of rods and spheres when DTX is encapsulated. When DTX is conjugated to the micelle core and crystallinity of the core increases, the morphology shifts to give only spherical micelles.
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uniform distribution with PI of 0.11. In contrast, the use of THF as the organic solvent produced larger micelles at 109 ± 29 nm that were also more disperse, having a PI = 0.52. Both of the above examples provide excellent guidelines for the selection of cosolvents for micellization.

Temperature influences inter-micelle chain movement as well. For example, the PDLLA core of PEG-PDLLA micelles had increased mobility at temperatures above their glass transition temperature (T_g). As a result, the CMC increases at greater temperatures demonstrating the direct impact of temperature on thermodynamic stability [69].

Micelle formation is an extremely sensitive process, susceptible to permeations from a number of internal and external sources. Even when a stable micelle is formed, there can be complications with lyophilization which suggest insufficient coverage of PEG or other hydrophilic component.

Kinetic stability

Kinetic stability describes the behavior of the micelle system over time in aqueous solution, specifically dealing with the dynamics between individual micelles, their environment and each other. Any change in the environment of a micelle can impact stability. As drug delivery vehicles, micelles are exposed to extreme and acute changes in their environment as described above. After micellization, individual chains remain dynamic and exchange between micelles and the bulk solution. Finally, after being exposed to changes in the environment or by simple dilution, micelles will begin to fall apart. Therefore, kinetic stability is used to describe the dynamics of micelles over time and during the process of disassembly. It is essential to characterize the kinetic stability of micelles to ensure that the encapsulated drug cargo is not released prematurely.

At equilibrium, the concentration of individual polymer chains (A) with respect to the concentration of micelles can be described as shown below:

$$K_M = \frac{[A]^n}{[micelle]}$$

K_M is the micelle dissociation constant and has the units of concentration; n is the aggregation number of the micelle.

As described by Mattice and co-workers, the dynamic equilibrium for copolymer exchange in micelles can be classified into three distinct mechanisms: chain insertion/expulsion, micelle merging/splitting, and micelle spanning (Fig. 5) [70]. For chain expulsion and insertion, a polymer chain is expelled from one micelle, returns to the bulk, and is then inserted into a second micelle. Chains can be exchanged when two micelles temporarily merge and the micellar cores come in contact. Here, the chain transfers from the first micelle to the second.

The final mechanism for copolymer exchange occurs by micellar spanning. The exteriors of two micelles are bridged by one extended chain without the chain ever completely returning to the bulk. The chain migrates from one micelle to another in this manner without being completely expelled from a micelle and without the two micelle cores ever contacting. Such elaborate kinetic studies can be modeled or observed experimentally by labeling one chain and tracing the transitions. Early work by Aniansson and Wall described

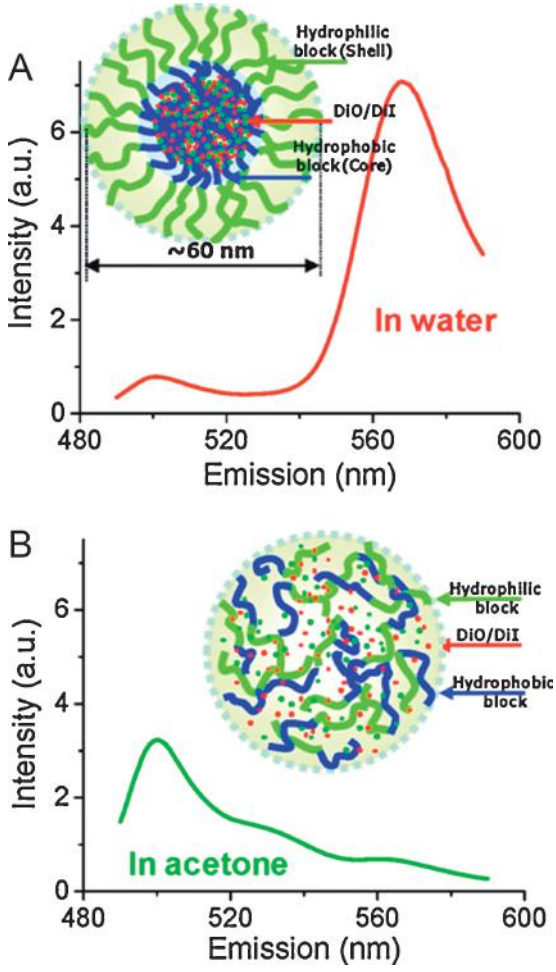

Figure 4 Micelles incorporating Fluorescence Resonance Energy Transfer (FRET) pair DiO and DiI are used to determine micelle stability. Representative fluorescence spectra showing micelles (A) assembled in water and (B) disassembled in acetone.
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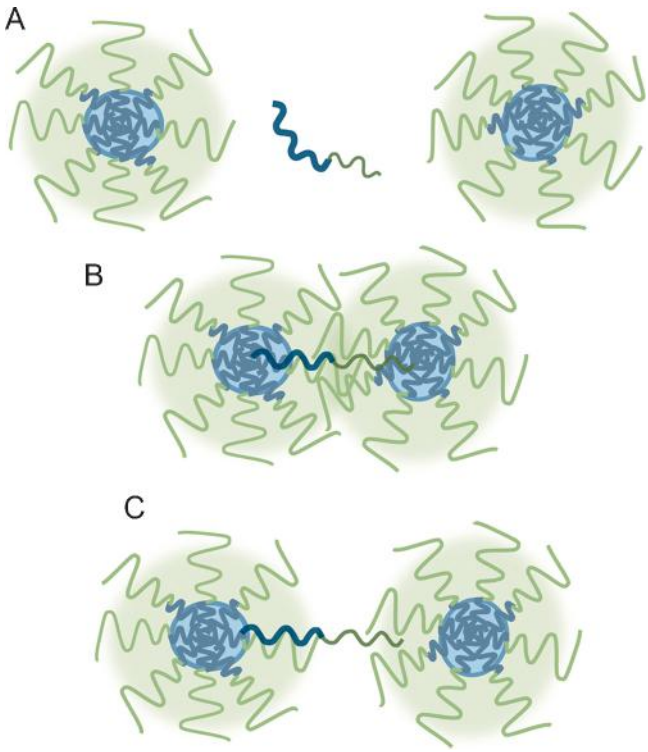

Figure 5 Methods for copolymer exchange in micelles can be classified into three distinct mechanisms: (A) chain insertion/expulsion, (B) micelle merging/splitting, and (C) micelle spanning.

the kinetics of micelle relaxation by polymer exchange and disassociation [71,72]. Recently, Diamant et al. have revisited kinetic modeling which described multiple stages for the micellization process [73].

Measuring kinetic stability

In general, the same analytical techniques used to measure thermodynamic stability can also be applied to observe kinetic stability. Most common among these are HPLC size exclusion chromatography (SEC) and dynamic light scattering (DLS). For example, Lin et al. used DLS to monitor a series of tri-block copolymers of PCL—PEG—PCL [74]. Micelles were incubated at 4 °C and the size measured each week for 8 weeks. Over the course of the study, micelles did not show significant levels of aggregation or disassembly.

Förster resonance energy transfer (FRET) experiments have recently been used to measure micelle integrity. Several groups have utilized FRET experiments using lipophilic fluorescent energy donor probes, DiO, and acceptor probes, DiI, loaded in the micelle core [75—77]. If the micelle is intact, FRET occurs between the donor probe (excited at a wavelength of 484 nm), and the acceptor probe resulting in fluorescence emission at 565 nm. Upon micelle dissociation, the distance between FRET pair is increased, resulting in decreased emission at 565 nm and increased emission at 501 nm (the emission of the donor probe) (Fig. 4) [47,77].

In an innovative approach, Yu et al. directly measured the force required to disassemble micelles in water using atomic force microscopy (AFM)-single molecule force spectroscopy

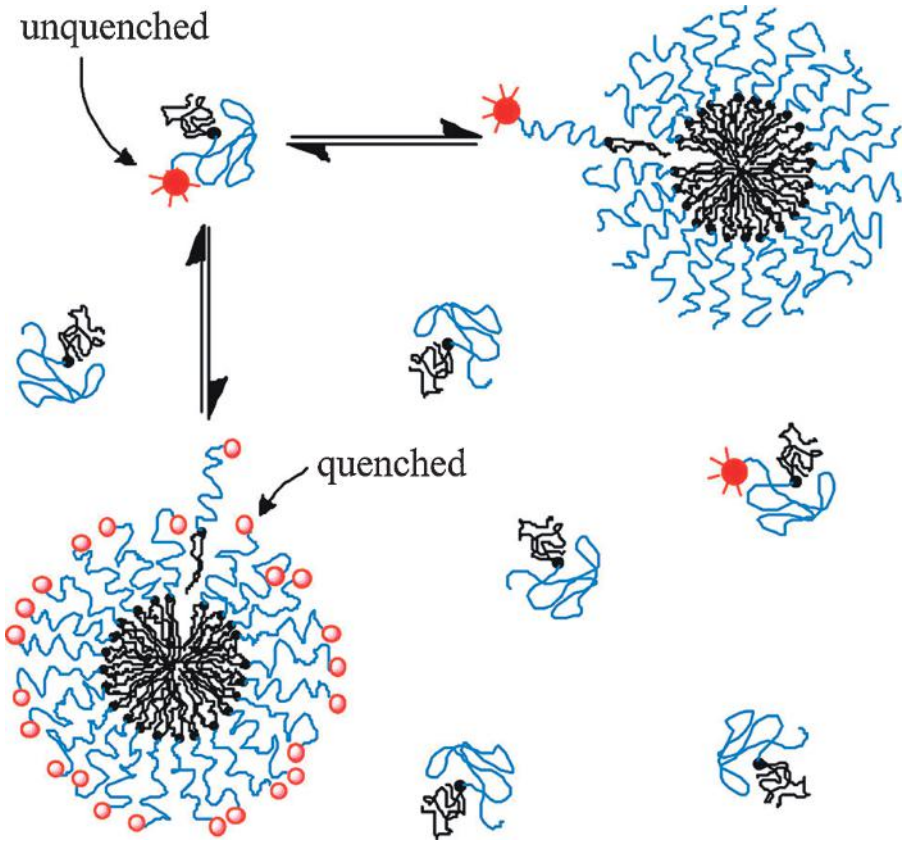

Figure 6 Monomer desorption rate is measured by fluorescence self-quenching between rhodamine-labeled polymers when micelles with high rhodamine surface density are mixed with unlabeled micelles. Rhodamine fluorescence decreases as quenched polymers (red circles) leave the densely labeled micelle, becoming unquenched (solid red hairy circles). Unquenched polymers are then free to remain in solution or incorporate into unlabeled micelles.
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(SMFS) [78]. Their results show that micelle disassembly depends strongly on solvent properties where the force required to disassemble micelles in water completely disappears in ethanol.

Kinetic stability stems from polymer chain exchange between micelles

Tirrell's lab measured the monomer desorption/exchange rates of micelles by labeling 1,2-distearoyl-phosphatidyl ethanolamine-PEG (2000) (DSPE-PEG2000) with rhodamine [79]. At high rhodamine surface density, fluorescence is self-quenched. Rhodamine-labeled micelles were mixed with unlabeled micelles and the increase in fluorescence was measured as the quenched polymers left the labeled micelle and were incorporated into unlabeled micelles or return to the bulk (Fig. 6). As such, the observed fluorescence intensity is a measurement of the rate of polymer desorption.

Merkx and Reulen furthered the investigation of polymer exchange by functionalizing DSPE-PEG2000 micelles with the FRET protein pair consisting of enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP) [80]. They determined that the number and rate of transitions is directly related to aggregate number and kinetic stability. Most importantly, the authors concluded that the short polymer exchange half-life of 4 min shows that this micelle system is not kinetically stable under physiologic conditions.

Kinetic stability is dependent on CMC, T_g, block ratios, and the encapsulated drug

In terms of CMC, Van Domeselaar et al. used poly(ethylene oxide-*block*-peptide) (PEO-*b*-polypeptide) to show that micelle stability is correlated strongly with core length. PEO-*block*-poly(tyrosine)$_{15}$ displayed less than 30% dissociation after seven days below the CMC while the shorter PEO-*block*-poly(tyrosine)$_{12}$ was less stable. Dissociation was observed by dynamic light scattering experiments [48].

Jette et al. altered the polycaprolactone (PCL) core length in poly(ethylene glycol)-*block*-polycaprolactone (PEG-*b*-PCL) copolymers. The dissociation of micelles was then measured by separating chains from intact micelles using HPLC and size exclusion. Their results confirmed that increasing the hydrophobic lengths in block copolymers resulted in increased kinetic stability [81].

A fluorescence based method, corroborated by surface tension measurements was used by Grant et al. to show that micelles below the CMC may still be kinetically stable [82]. Alternatively, size exclusion HPLC can be used to measure the dissociation rate of micelles below the CMC. The elution

times of polymer chains and micelles can therefore be used to evaluate micelle integrity [48].

Stability is directly related to the hydrophobic character of the core. The hydrophobicity of the core can be modified by increasing chain length or chemical modification. Strategies to improve stability include altering hydrophobic and hydrophilic segment lengths or altering the hydrophobicity of the core [58,69,83–85]. Studies from the Kwon lab showed that poly(ethylene oxide)-*block*-poly(*N*-hexyl-L-aspartamide) (PEO-*b*-p(*N*-HA) micelles had more fluid core regions than PEO-*b*-p(*N*-HA) micelles derivatived to incorporate stearate side chains. Increasing the degree of stearate esterification increased the hydrophobic interactions in the core and resulted in micelles with greater thermodynamic and kinetic stability [58,83]. In a separate approach, Thurmond et al. found that cross-linking the core of hydrophobic micelles, after assembly, also increases stability [85].

Destabilization from environmental factors

Micelle stability also depends on the composition and concentration of disrupting agents in solution. Work by Savic used fluorescein-5-carbonyl azide conjugated to the micelle core of poly(caprolactone)-*block*-poly(ethylene oxide) (PCL-*b*-PEO) to evaluate the integrity of micelles in media of varying complexity. Micelles were stable in phosphate buffered saline but disassembled in serum and multicomponent media [84]. The authors did not elucidate the specific source of micelle destabilization.

The hydrophobic core of micelles is protected from the aqueous environment by the hydrophilic corona [86]. If, however, the hydrophobic components are exposed to the aqueous medium, the micelle loses integrity [87,88]. Altering the molecular weight ratio between the hydrophobic and hydrophilic segments of the copolymer allows it to resist destabilization by the environment. For example, when hydrophilic PEG is bound to strongly hydrophobic polystyrene (PS) segments, even the addition of surfactants does not destabilize the micelles [88]. Yokoyama and colleagues showed that adriamycin-conjugated poly(ethylene glycol)-poly(aspartic acid) (PEG-P[Asp(ADR)]) micelles were stable in distilled water but lost their integrity in phosphate-buffered saline under similar conditions [89]. The difference between these two systems is the strong hydrophobic interactions between the poly(styrene) chains that are absent in the poly(aspartic acid) chains as a result of the environment — poly(aspartic acid) contains ionizable groups that respond to the pH and osmolarity of the solution.

In addition to changes in salt content and pH in solution composition, micelles are exposed to a vast array of proteins following systemic delivery. Proteins can adsorb to the surface of micelles and disrupt micelle cohesion and stability [50]. Three mechanisms of micelle instability resulting from their interaction with serum proteins have been proposed: protein adsorption, drug extraction, and protein penetration into the core [90]. Protein adsorption to functionalized micelles was shown by Thayumanavan and co-workers to cause destabilization and subsequent release of guest molecules from the micelle [91]. Lynch et al. investigated the interaction of micelles and two of the most abundant serum proteins, albumin and fibrinogen [92]. The authors demonstrate that both of these proteins do indeed bind dynamically to the surface of N-isopropylacrylamide (NIPAM) and N-tert-butylacrylamide (BAM) based polymeric micelles.

Consistent with the Vroman effect, recent work suggests that many proteins interact with micelles and other nanoparticles in a dynamic fashion and with a range of affinities. An important, emerging concept is that of a ''protein corona'' that results from adsorption to the micelle surface and subsequently determines the biological response, including elimination *via* the mononuclear phagocyte system (MPS) and uptake into cells. The reader is directed to a recent review that covers this topic in detail [93]. Considering the plethora of PEG-based micelles, the emerging evidence of protein adsorption and compliment activation of this polymer is profoundly important [94]. Alternate hydrophilic polymers are required to overcome some of the limitations posed by PEG.

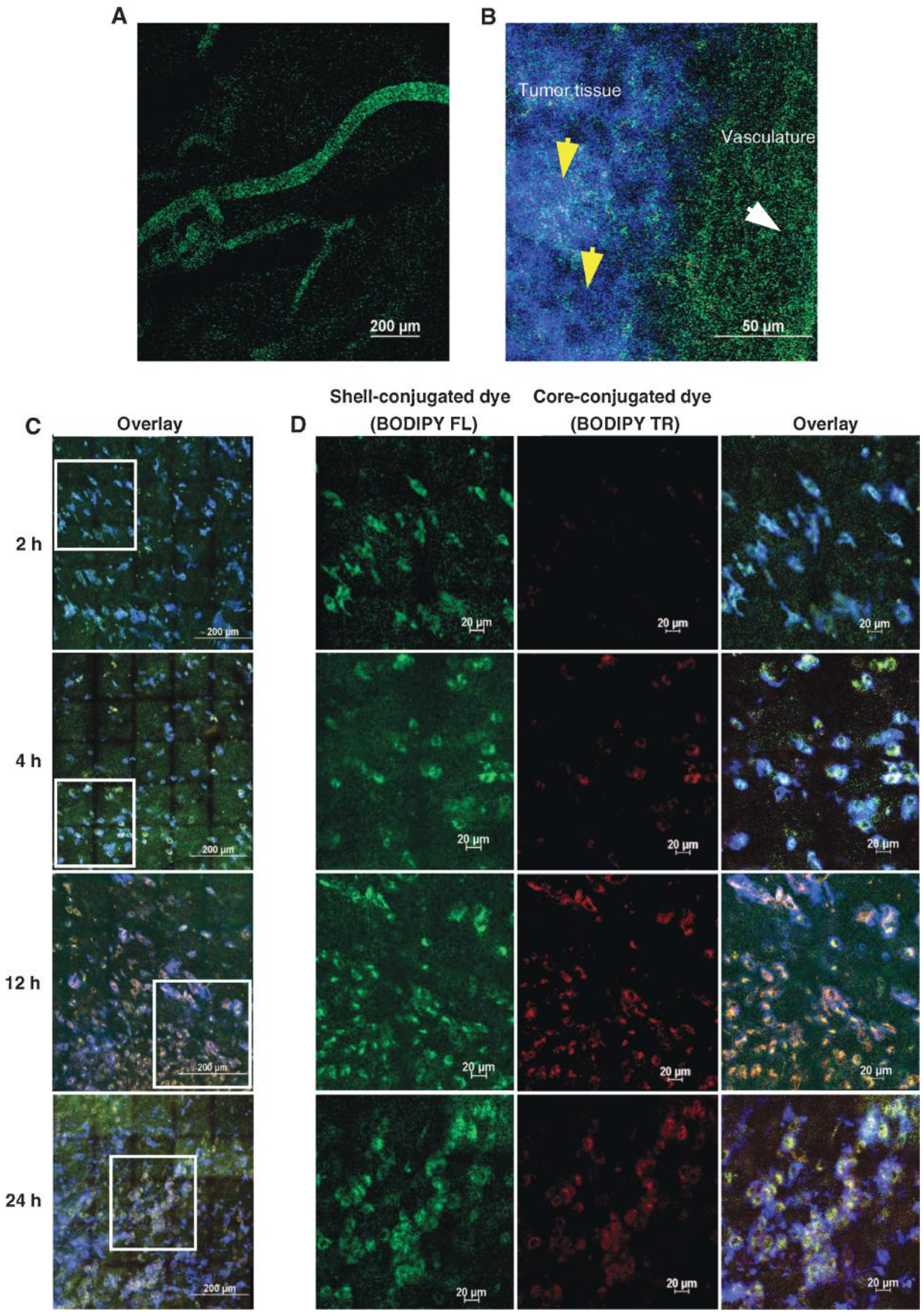
Stability under physiologic conditions

Much insight has been gained from the micelle stability studies mentioned above. Nevertheless, it has become apparent that in order to fully understand the implications of micelle stability on effective drug delivery, we must consider micelles under more representative conditions.

FRET experiments utilizing DiO and DiI as the FRET donor and receptor, respectively, have been used to evaluate micelle stability in the presence of serum proteins. Kwon and co-workers showed that PEG-*block*-poly(*N*-hexyl stearate L-aspartamide) (PEG-*b*-PHSA) micelles were stable in serum albumin, alpha and beta globulins, and gamma globulins for 1–2 h [47]. Chen et al. investigated (PEG-DSPE) micelles and found they were *not* stable in serum after injection [75]. Using a similar FRET approach, as well as SEC, the Shoichet lab showed that poly(DL-lactide-co-2-methyl-2-carboxytrimethylene carbonate)-*graft*-PEG (poly(LA-co-TMCC)-*g*-PEG) micelles had a half-life of ~9 h in complete serum and were even more stable when exposed to individual serum proteins [77]. The micelle diameter and polydispersity index did not change significantly after incubation with albumin, suggesting only limited albumin absorption.

The Maysinger lab conjugated a fluorogenic probe to the hydrophobic segment of PEG-*b*-PCL micelles [84]. Intact micelles were nonfluorescent as a result of autoquenching of the fluorophore, whereas fluorophores on disassembled micelles are not quenched and therefore showed measurable fluorescence. Micelles incubated for 48 h in phosphate buffer showed no change in fluorescence levels; however, incubation in RPMI media with no serum showed a 13% increase in fluorescence and incubation in RPMI with 5% serum showed a 64% increase in fluorescence after 48 h, demonstrating increased instability of micelles with increasingly relevant protein concentrations.

Camptothecin (CPT) was used to evaluate the stability of PEG-poly(benzyl aspartate) by Okano and colleagues [95]. CPT was encapsulated into the micelle core and the rate of hydrolysis of the CPT lactone ring was measured. As micelles disassembled, more CPT escaped and the rate of lactone hydrolysis increased. Micelles were significantly destabilized in the presence of human and bovine serum and human


A
200 μm
B
Tumor tissue
Vasculature
50 μm
C
Overlay
2 h
4 h
12 h
24 h
200 μm
D
Shell-conjugated dye
(BODIPY FL)
Core-conjugated dye
(BODIPY TR)
Overlay
20 μm

albumin; however, mouse albumin *did not* affect the stability of micelles. Considering the common progression from *in vitro* studies to initial preclinical evaluation in mice, this result is of utmost importance and shows the foreboding challenge of extrapolating animal models to clinical applications.

Kataoka and co-workers designed a double-labeled micelle to measure *in situ* micelle integrity by fluorescence [96]. Two distinct fluorophores were conjugated to PEG-*block*-poly(glutamic acid) PEG-*b*-poly(Glu) micelles — one on the corona surface and one in the core. The core conjugated dye is quenched if the micelle remains intact and thus only the corona conjugated dye emits fluorescence. When micelle integrity is lost, the fluorescence of the core-conjugated dye is visible. This system was used to evaluate the cellular uptake of the micelles. Most significantly, these micelles were also utilized to monitor the *in vivo* distribution and integrity of micelles in blood vessels and tumors after intravenous administration (Fig. 7), thereby providing important insight into the mechanism of circulation half-life and tumor targeting. The authors show that micelle-carried drug may bypass the normal cell defense mechanisms, potentially increasing the amount of drug delivered to otherwise resistant cells.

Each of the above examples provides excellent starting points to investigate the stability of micelles in relevant media and represent the cutting-edge of studying micelle stability; however, the contribution of the various ''additives'' should not be overlooked. For example, in the FRET experiments outlined, both DiO and DiI are incorporated into the micelles and therefore have a direct impact on the structure/stability of the system. Likewise, 'Impact of encapsulated and conjugated drug' section outlined the influence of encapsulated and conjugated drugs on the stability of micelle systems. Pendant fluorophores, such as those mentioned here, often have many drug-like characteristics and as such will also influence the micelles they are used to probe. Investigators should be cautioned to either develop methods to investigate micelle stability without perturbing the system, or use modified micelles in final formulations.

Conclusions and outlook

Over the past 30 years, polymeric micelles have become increasingly sophisticated in their design; however, many micelles are not evaluated in physiologically relevant media, thereby limiting the predictability of the *in vitro* assay to the complex *in vivo* microenvironment. The scientific community has benefited from the investigation of micelle stability in water or aqueous buffers, revealing mechanisms of both micellization and destabilization. The contributions of individual micelle components have been elucidated and can be further tuned to increase the thermodynamic and kinetic stability of micelles used for drug delivery.

Understanding micelle stability in physiologically relevant media is the first step to establishing whether a micelle has promise for drug delivery. The most relevant media is human blood with its full complement of proteins and cells. The difficulty with using this complex system lies in establishing a mechanistic understanding of the results. To gain a greater understanding, the media can be broken down into individual components. When examining micelle stability, a good place to start is serum and the major protein constituents therein (*e.g.* albumin, globulins). Micelle stability can be further examined with the addition of cells. Advancing from the bench top to the animal model is critical to gain further insight to micelle stability and will likely require that new methods be developed to answer fundamental questions which cannot be answered using fluorescence techniques to the level of sensitivity required.

There are a significant number of unanswered questions related to micelle stability. For example, if the hydrophobic drug interacts with the hydrophobic core, then how is it released and how does this drug release influence micelle stability? Moreover, if drug loading is a limitation, how will can it be enhanced and in turn how will this affect micelle stability? Additional questions relate to micelle stability during freeze-drying and re-constitution. These impact scalability and development of micelles for clinical use.

PEG has been studied almost exclusively as the hydrophilic component of amphiphilic copolymer micelles with a variety of hydrophobic cores. There is clearly a significant opportunity for the investigation of new hydrophilic components. The need for new hydrophilic polymers is substantiated by the mounting evidence that, contrary to popular belief, proteins bind to the surface of micelles and destabilize them. Micelle stability may be extended by preincubating micelles in specific proteins prior to i.v. injection as a way to alter the biologic response.

Micelle stability may be further enhanced by crosslinking the core or the corona [97]. In achieving a more stable micelle, we must evaluate the effect of crosslinking on drug release and cellular interaction. The physical properties of the micelle may also impact its ability to cross the hyperpermeable vasculature that surrounds tumor tissue. Therefore, micelle stability cannot be isolated from drug delivery.

Importantly, current micelle systems have progressed beyond copolymer backbone design to include conjugated targeting molecules. These modifications can potentially increase the targeting of micelles to diseased sites, as well

Figure 7 *In vivo* confocal laser scanning microscopy (CLSM) observation of PEG-b-poly(Glu) dual fluorescence micelles in blood vessels and tumors after intravenous administration. (A and B) CLSM observation of micelle in the blood vessels of solid tumors (A) immediately after injection and (B) in the tumor tissue at 12 h after injection. Yellow arrows, tumor tissue; white arrow, blood vessel. (C) Time-dependent CLSM observation of fluorescent micelles in the tumor tissues at 2, 4, 12, and 24 h after injection. Green, fluorescence from the shell-conjugated dyes (BODIPY FL); red, core-conjugated dyes (BODIPY TR); blue, cell surfaces stained by CellMask. (D) Magnification of selected areas [square regions in (C)] by channel.
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as boost the binding to cells and subsequent internalization [98]. Notwithstanding these important benefits, the effect of having a targeting ligand exposed to blood serum proteins and cells may also influence micelle stability and *in vivo* circulation in blood.

A new trend in micelle research is the co-delivery of both therapeutic and diagnostic agents. These ''theranostic'' systems boast two benefits in a single macromolecule where the therapeutic is specifically delivered to the desired site while the diagnostic images the same tissue, thereby providing greater information on the cancer tissue [99]. There is significant excitement surrounding these systems but they, like the simple micelles, need to be studied in relevant media — both for micelle stability, cellular interaction and ultimately efficacy in relevant animal models with orthotopic (and not ectotopic or subcutaneous) tumors.

Numerous possibilities exist to expand micelle chemistry and stability. With each modification, the system becomes more complex and the evaluation of these systems must be equally sophisticated to provide meaningful results. A more thorough understanding of polymeric micelle stability under physiological conditions will facilitate the ultimate clinical success — greater localized therapeutic efficacy and reduced systemic toxicity.

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