

Determination of critical micelle concentrations and aggregation numbers by fluorescence correlation spectroscopy: Aggregation of a lipopolysaccharide

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Abstract

Fluorescence correlation spectroscopy (FCS) is often used to determine the mass or radius of a particle by using the dependence of the diffusion coefficient on the mass and shape. In this article we discuss how the particle size of aggregates can be measured by using the concentration dependence of the amplitude of the autocorrelation function (ACF) instead of the temporal decay. We titrate a solution of aggregates or micelles with a fluorescent label that possesses a high affinity for these structures and measure the changes in the amplitude of the ACF. We develop the theory describing the change of the ACF amplitude with increasing concentrations of labels and use it to fit experimental data. It is shown how this method can determine the aggregation number and critical micelle concentration of a standard detergent nonaethylene glycol monododecyl ether (C₁₂E₉) and a lipopolysaccharide (LPS: *Escherichia coli* 0111:B4).

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1. Introduction

Lipopolysaccharide (LPS, also known as endotoxin) is the major component of the outer membrane in Gram-negative bacteria and it can activate the host defense system of most organisms. However, it is also known that LPS is responsible for excessively stimulating the production of various inflammatory cytokines which are secreted by host monocytes and macrophages. The excessive cytokines will eventually cause organ failure and death after Gram-negative bacterial sepsis [1–4]. Therefore the characteristics of LPS have been widely investigated. LPS which is negatively charged is composed of three parts: O-antigen, polysaccharide and lipid A [5] (Fig. 1C). Lipid A which consists of six or seven hydrocarbon chains and a β -D-GlcN-(1-6)- α -D-GlcN disaccharide with two phosphoryl groups is the conserved bioactive component of LPS which is responsible for most of the endotoxic effects [6]. The lipid A moiety is the most conserved part between LPS of differ-

ent serotypes. The polysaccharide is the linker between lipid A and O-antigen and is constituted by two cores. The inner core is mainly composed of 3-deoxy-D-manno-octulosonic acid (Kdo) and L-glycero-D-manno heptose (Hep), and the outer core includes hexoses and hexosamines [7]. Lipid A and Kdo are prerequisite for forming a minimum LPS molecule [8]. O-antigen which is an important surface antigen extends into the environment [6,9]. It consists of 0–50 repeating subunits which are composed of 1–8 glycosyl residues [7]. For different bacterial serotypes, there are great differences in the length and composition of the polysaccharide and O-antigen.

Although the structure and pathophysiological functions of LPS have been extensively studied, the physical property such as aggregation of LPS in aqueous solution is not well known. LPS which is an amphipathic molecule with hydrophobic part (lipid A) and hydrophilic group (O-antigen) might act as a detergent in aqueous solution forming micelles when it reaches a threshold concentration, referred to as critical micelle concentration (CMC). At present, many techniques such as light scattering spectroscopy and fluorescence spectroscopy are used to determine the CMC of LPS micelles [6,9]. In this paper we apply fluorescence correlation spectroscopy (FCS)

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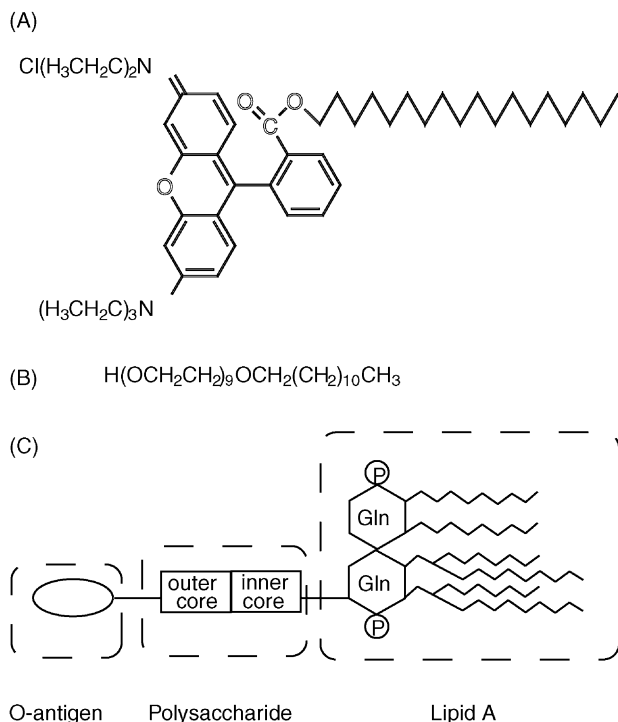


Fig. 1. (A) Structure of octadecyl rhodamine B chloride (R18). The long hydrophobic alkyl chain helps incorporation into micelle. (B) Formula of C_{12}E_9 . (C) Structure of LPS, consisting of lipid A, polysaccharide and O-antigen.

to explore the aggregation of LPS. FCS which is a technique with single molecule sensitivity measures the average number of independently moving fluorescent particles in a confocal observation volume and their average diffusional transition time through that volume. From these two parameters the concentration and diffusion coefficient of a particle can be calculated.

Up to now mainly the diffusion time, and thus the diffusion coefficient, was used to determine molecular weights [10–12]. However, although it has been shown that the method can be accurate for certain systems [12], this method has several inherent problems. Firstly, the diffusion coefficient depends only weakly on the mass (for spherical particles $D \sim R^{-1}$, and thus it is assumed $D \sim M^{-1/3}$). Therefore, a 10% error in the diffusion coefficient will result in a 30% error in mass. Secondly, the diffusion coefficient actually measures the hydrodynamic radius which exceeds the actual particle radius and thus leads to an overestimation of the particle mass [13]. Thirdly, in the absence of exact data on shape and density a spherical shape and constant density of particles have to be assumed. Any deviation of the real system from these assumptions leads to biased measurement. In this work we titrate micelles with a hydrophobic fluorescent probe, which strongly partitions into the micelles. By using the number of labeled particles in FCS instead of the diffusion time we can directly determine the CMC and the aggregation number (N_{agg}). This method is successfully applied to the well-known detergent nonaethylene glycol monododecyl ether (C_{12}E_9) and is then used to determine N_{agg} of LPS *Escherichia coli* strain 0111:B4.

2. Materials and methods

2.1. Materials

Lipopolysaccharide from *Escherichia coli* strain 0111:B4 (LPS), its fluorescent derivative fluorescein isothiocyanate lipopolysaccharide (FITC-LPS), nonaethylene glycol monododecyl ether (C_{12}E_9), Triton-X100 and phosphate-buffered saline (PBS, pH 7.4) were purchased from Sigma-Aldrich (Sigma-Aldrich Pte Ltd., Singapore). Octadecyl rhodamine B chloride (R18) is a product from Molecular Probes (ITS Science and Medical Pte Ltd., Singapore).

2.2. FCS instrumentation

Two different instruments were used in this study. For experiments involving R18 a commercial laser scanning confocal microscope (FV300, Olympus, Singapore) was modified and combined with FCS. For the FV300, a HeNe laser (543 nm, Melles Griot, Singapore) is coupled into the scanning unit after passing through an optical fiber, and reflected by a mirror and an excitation dichroic mirror (488/543/633) into a pair of galvanometer scanning mirrors (G120DT, GSI Lumonics, Singapore). After being scanned, the laser beam is directed into a water immersion objective (60 \times , NA1.2, Olympus, Singapore) by a reflective prism and focused into a small focal volume in the specimen. The fluorescence emitted from the sample is collected by the same objective, descanned and focused again by a collecting lens into a confocal pinhole. A glass slab is used for light beam xy position alignment. A modified detection part for FCS was mounted on the top of the scanning unit. The fluorescence light after the confocal pinhole is imaged by a lens (Achromats $f=60$ mm, Linos, Goettingen, Germany), through an emission filter (580DF30, Omega, Brattleboro, VT, USA), into the active area of a photomultiplier tube (PMT, H6240-01, Hamamatsu Photonics K.K., Japan) and the signal was autocorrelated by a digital correlator (Flex02-12D, <http://www.correlator.com>). The program IgorPro (Wavemetrics, Lake Oswego, OR, USA) was used for fitting of the autocorrelation function to experimental data as described previously [14].

For experiments on FITC-LPS we used a self-built fluorescence correlation spectrometer centered around an Axiovert 200 inverted microscope (Carl Zeiss South East Asia, Singapore). The excitation radiation of 488 nm provided by an Argon laser (Melles Griot SP, Pte Ltd., Singapore) first passed through a beam expansion and was focused on the samples using a water immersion objective (C-Apochromat, 63 \times , NA 1.2, Zeiss). A dichroic filter and an emitter (505DRLP and 530DF30, Omega) were used to separate the excitation light from the emission fluorescence. A 50 μm diameter pinhole in the image plane blocked out-of-focus signals. The emitted fluorescence was detected by a photomultiplier tube (PMT, H6240-01, Hamamatsu Photonics K.K., Japan) and the signal was autocorrelated by a digital correlator (Flex02-12D, <http://www.correlator.com>).

In all experiments the laser power was set to 40 μW before entering the microscope and FCS measurements were taken for 10 to 30 s.

2.3. Preparation of different concentrations of C₁₂E₉ micelle with R18

C₁₂E₉ was weighed and dissolved in PBS to prepare a stock solution of 0.20 mM. The stock solution was diluted to 0.04, 0.05, 0.06, 0.07, 0.08, 0.09, 0.10, 0.12, 0.14, 0.16, 0.18, 0.20 mM and each solution was mixed with 20 or 40 nM R18 solutions. The mixed solutions were incubated at least 2 h followed by FCS experiments at room temperature.

2.4. Preparation of C₁₂E₉ micelle with different concentrations of R18

Stock solutions of 0.15 mM C₁₂E₉ were prepared in individual vials and mixed with 10, 20, 50, 75, 100, 150, 200, 250, 300, 350 and 450 nM R18. The mixtures were incubated for at least 2 h before FCS experiments.

2.5. Preparation of different concentrations of LPS micelle with R18

Different concentrations of LPS were obtained by diluting a stock solution of LPS (100 μM) to low concentration using PBS. A series of LPS solutions, from 250 nM up to 5 μM, were mixed with 10 or 20 nM R18 solution. FCS experiments were done after at least 2 h incubation.

2.6. Preparation of LPS micelle with different concentrations of R18

LPS solutions of 2 μM were prepared and mixed with 1, 5, 10, 15, 20, 30, 40, 50 and 60 nM R18. Mixtures were incubated at least 2 h followed by FCS experiments at room temperature.

2.7. Interaction of FITC-LPS and Triton-X100

FITC-LPS of the following concentrations, 1, 5, 10, 15, 20, 30 and 50 μM was diluted in PBS in individual vials and analyzed. A separate set of FITC-LPS of 1, 5, 10, 15, 20, 30 and 50 μM was diluted in PBS. 17 mM Triton-X 100 (CMC = 0.24 mM) dissolved in PBS was added and the mixture was vortexed and analyzed. Samples were measured at room temperature.

3. Theory

3.1. Titration of a solution of aggregates with a fluorescent probe

The concentration of micelles in a detergent solution is given by the concentration of detergent molecules c_{det} less the concentration of detergent molecules which exists as monomers, c_{cmc} , divided by the number of detergent molecules per micelle N_{agg} :

$$c_{\text{mic}} = \frac{c_{\text{det}} - c_{\text{cmc}}}{N_{\text{agg}}} \quad (1)$$

This is a common definition that assumes that the detergent solution undergoes an abrupt change from a monomeric solution to

a solution containing micelles exactly at c_{cmc} . In how far this is justified we will discuss in the experiments. N_{agg} is to be understood as an average value and the actual number of molecules per micelle can vary around this value. From this the number of detergent micelles in an observation volume can be calculated:

$$N_{\text{mic}} = c_{\text{mic}} V_{\text{obs}} N_A \quad (2)$$

If the concentration of fluorescent probe molecules is given by c_{pr} , then on average one has m probe molecules per micelle:

$$m = \frac{c_{\text{pr}}}{c_{\text{mic}}} = \frac{N_{\text{agg}} c_{\text{pr}}}{c_{\text{det}} - c_{\text{cmc}}} \quad (3)$$

Assuming that binding of the probe molecules to micelles is not cooperative, the probability to find a micelle with k probes attached is given by a Poisson distribution:

$$P(k, m) = \frac{m^k e^{-m}}{k!} \quad (4)$$

the probability to find micelles without a label is given by

$$P(0, m) = e^{-m} \quad (5)$$

and the overall number of micelles with at least one label is

$$N = N_{\text{mic}}(1 - P(0, m)) \approx N_{\text{mic}} \sum_{k=1}^L \frac{m^k e^{-m}}{k!} \quad (6)$$

We have here approximated the Poisson distribution by a finite sum since in FCS each factor in the sum will have to be evaluated individually due to differences in the count rate per particle as will be explained below (see Eqs. (11)–(19)). The cut-off L in this sum was set arbitrarily at the number of labels k for which the probability $P(k, m) \leq 0.01$. For instance, for $c_{\text{pr}} = 3c_{\text{mic}}$, i.e. the concentration of labels is three times higher than the micelle concentration, $L = 8$ since the probability to find a micelle with more than eight labels is smaller than 1%.

The number N as defined in Eq. (6) is the observable parameter in FCS. In the following we distinguish two different cases depending on the ratio of $c_{\text{pr}}/c_{\text{mic}}$. If measuring at probe concentrations much below the concentration of micelles $c_{\text{pr}} < 0.1 c_{\text{mic}}$ then the sum over all $P(k, m)$ with $k > 1$ is smaller than 0.5% and particles with more than one label can be neglected. Then it follows from Eqs. (3), (5) and (6) that

$$N = N_{\text{mic}}(1 - e^{-c_{\text{pr}}/c_{\text{mic}}}) \quad (7)$$

Using Eqs. (1) and (2) this can be written as a function depending on the probe label, the detergent concentration, CMC and N_{agg} :

$$N = \frac{c_{\text{det}} - c_{\text{cmc}}}{N_{\text{agg}}} V_{\text{obs}} N_A (1 - e^{-c_{\text{pr}} N_{\text{agg}} / (c_{\text{det}} - c_{\text{cmc}})}) \quad (8)$$

In the case for $c_{\text{pr}} \geq 0.1 c_{\text{mic}}$ the different $P(k, m)$ cannot be neglected anymore and have to be included. Eqs. (1)–(3) and (5) and (6) yield the following equation for the number of labeled particles:

$$N = \frac{c_{\text{det}} - c_{\text{cmc}}}{N_{\text{agg}}} V_{\text{obs}} N_A \sum_{k=0}^L \frac{\left(\frac{N_{\text{agg}} c_{\text{pr}}}{c_{\text{det}} - c_{\text{cmc}}} \right)^k e^{-N_{\text{agg}} c_{\text{pr}} / (c_{\text{det}} - c_{\text{cmc}})}}{k!} \quad (9)$$

Eqs. (8) and (9) determine the average number of labeled particles in any observation volume. This number can be measured with FCS as discussed in the next section.

3.2. Fluorescence correlation spectroscopy (FCS)

FCS is a biophysical technique with single molecule sensitivity. It analyzes fluorescence intensity fluctuations caused by minute deviations from thermal equilibrium from a confocal volume in a sample which contains fluorescent particles [15,16]. These fluctuations are caused by different molecular processes such as chemical reactions, rotational diffusion, translational diffusion, flow and binding processes. Fluctuations in the fluorescence signal are quantified by temporally autocorrelating the recorded intensity signal [17,18]. The fluorescence intensity autocorrelation function (ACF) is calculated by

$$G(\tau) = \frac{\langle F(t)F(t+\tau) \rangle}{\langle F(t) \rangle^2} = \frac{\langle \delta F(t)\delta F(t+\tau) \rangle}{\langle F(t) \rangle^2} + 1 \quad (10)$$

The angular brackets describe a time average, $F(t)$, the fluorescence signal as a function of time, τ , the correlation time and $\delta F(t)$ is the fluorescence fluctuations around the mean value. The general solution for confocal FCS taking account of different species that undergo free diffusion including possible triplet states is

$$G(\tau) = \frac{1}{N} \frac{\sum_{k=1}^L \alpha_k^2 F_k g_k(\tau)}{\left[\sum_{k=1}^L \alpha_k F_k \right]^2} + G_\infty \quad (11)$$

with

$$g_k(\tau) = \left(1 + \frac{4D_k\tau}{\omega^2} \right)^{-1} \left(1 + \frac{4D_k\tau}{z^2} \right)^{-1/2} \left(\frac{T_k e^{-\tau/\tau_{T_k}}}{1 - T_k} + 1 \right) \quad (12)$$

N is the average number of particles in the observation volume. It should be noted that the observation volume V_{obs} in this definition is characteristic for a setup and can vary with alignment. The observation volume, including any corrections due to the particular excitation intensity distribution in the observation volume, is ideally determined by a standard solution of defined concentration at the beginning of every experiment [19]. The coefficient α_k is the ratio of the fluorescence yield Q_k of particle k (in photon counts per particle and second) to the fluorescence yield Q_1 of particle 1:

$$\alpha_k = \frac{Q_k}{Q_1} \quad (13)$$

The fluorescence yields Q_k are the product of the extinction coefficient, quantum yield and overall photon detection efficiency for particle k in the instrument.

The coefficients F_k are the mole fraction of species k in the sample. G_∞ is the convergence value of the ACF for long times; from Eq. (1) it can be seen that G_∞ should be 1. D_k is the diffusion coefficient of species k , and ω and z are the radial and axial distances of the confocal volume at which the intensity has dropped by $1/e^2$ of the maximum intensity. T_k is the average

fraction of particles of species k that reside in the triplet state and τ_{T_k} is the characteristic decay time of the particles in the triplet state.

For later discussions we introduce here the average counts per particle and second (cps) given by the overall count rate multiplied by the amplitude of the correlation function $G(0) - G_\infty$. If only one particle species is present in solution the cps is a direct measure of the fluorescence yield Q_k since the amplitude $G(0) - G_\infty$ is proportional to $1/N$ (Eq. (11) for $L=1$ and $\tau=0$). If several particle species are in solution the cps is an average value for the different species. Changes in this value can indicate whether fluorescence yields change due to incorporation of several labels into one particle or if aggregation happens.

In this work we are interested exclusively in the amplitude of the correlation function, i.e. $\tau=0$, since it contains the information of the concentration of labeled particles. Thus Eq. (11) simplifies to

$$G(\tau) = \frac{1}{N} \frac{\sum_{k=1}^n \alpha_k^2 F_k \left(\frac{1}{1-T_k} \right)}{\left[\sum_{k=1}^n \alpha_k F_k \right]^2} + G_\infty \quad (14)$$

The number of particles in the FCS observation volume thus is

$$N = \frac{1}{G(0) - G_\infty} \frac{\sum_{k=1}^n \alpha_k^2 F_k \left(\frac{1}{1-T_k} \right)}{\left[\sum_{k=1}^n \alpha_k F_k \right]^2} \quad (15)$$

Eq. (15) represents the general solution for the measured number of fluorescent particles under different conditions and can be used to determine the number of labeled micelles. In the case that only one label is present on each micelle or the α_k are all equal, independent of the number of labels, Eq. (15) reduces to

$$N = \frac{1}{G(0) - G_\infty} \left(\frac{1}{1 - T_k} \right) \quad (16)$$

3.3. Fitting of FCS data to the expected number of labeled micelles

For micelles the different fractions F_k in Eq. (15) are given by

$$F_k = P(k, m) \quad (17)$$

The different α_k depend on the particular situation. In general, they have to be measured and cannot be easily recovered from fits to the ACF. We will discuss here two exemplary cases applicable to our situation. In the simplest situation all α_k are equal, which is e.g. the case if $c_{\text{pr}} \ll c_{\text{mic}}$. In this case the number determined by FCS does not have to be corrected for differences in α_k and Eq. (16) gives the experimentally determined number of micelles which can be fitted by the simple model of Eq. (8).

In the second case α_k is equal to the number of labels:

$$\alpha_k = k \quad (18)$$

This could be the case when the aggregates are so large that there is no interaction between the labels. Then a labeled micelle with two labels is exactly twice as bright ($\alpha_k = 2$) as a micelle with one label and according to Eq. (15) will contribute four times ($\alpha_k^2 = 4$) as much to the correlation function. The full model

has to be used including multiply labeled particles and taking account of triplet states. In this case the number of particles measured in FCS is given by

$$N = \frac{N_{\text{agg}}}{(c_{\text{det}} - c_{\text{cmc}})V_{\text{obs}}N_A} \times \frac{\sum_{k=1}^n \alpha_k^2 \frac{(N_{\text{agg}}c_{\text{pr}}/c_{\text{det}} - c_{\text{cmc}})^k}{k!} e^{-N_{\text{agg}}c_{\text{pr}}/c_{\text{det}} - c_{\text{cmc}}} \left(\frac{1}{1-T_k}\right)}{\left[\sum_{k=1}^n \alpha_k \frac{(N_{\text{agg}}c_{\text{pr}}/c_{\text{det}} - c_{\text{cmc}})^k}{k!} e^{-N_{\text{agg}}c_{\text{pr}}/c_{\text{det}} - c_{\text{cmc}}}\right]^2} \quad (19)$$

In this work this model is fitted to the experimental values with c_{cmc} , N_{agg} and V_{obs} , as free parameters, c_{det} and T_k (as determined from the fit to the experimental ACF) as fixed parameter, and the concentration of fluorescent labels c_{pr} as independent variable. Sample curves of the models for different values of $(c_{\text{det}} - c_{\text{cmc}})/N_{\text{agg}}$ are given in Figs. 2 and 3.

3.4. Disaggregation of FITC-LPS with a detergent

Another possibility to measure the number of LPS molecules in LPS micelles is to determine the change of the amplitude of the ACF when a fluorescently labeled LPS is dissolved by a detergent. Assuming that all LPS micelles have roughly the same fluorescence yield and that after the dissolution of the LPS micelles only labeled monomers in detergent micelles are found, one can directly calculate the number of LPS molecules per LPS micelle. If the relative fraction R of all LPS molecules is fluorescently labeled, the number of LPS molecules per LPS micelle is N_{LPS} , the number N_0 of the LPS in solution and the number N_1 of the same solution after dissolution with a detergent are related by

$$N_{\text{LPS}} = \frac{N_1}{RN_0} \quad (20)$$

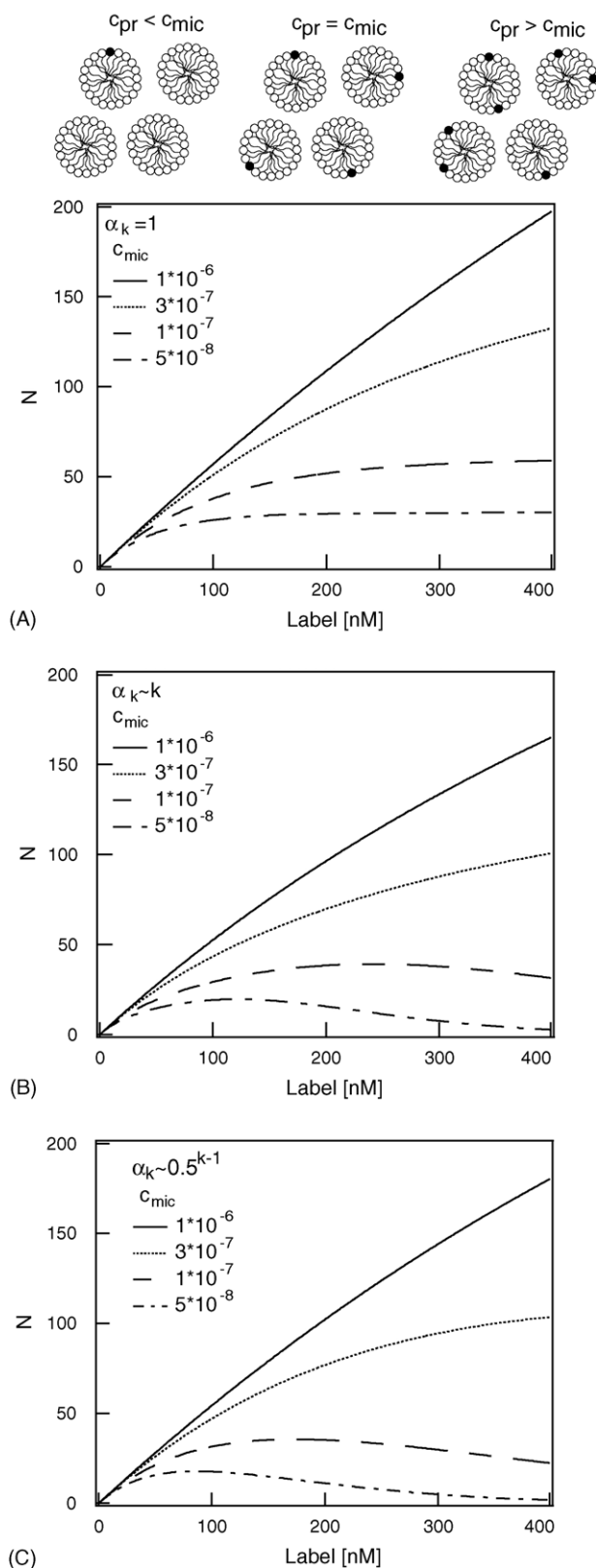
It should be noted that if the assumption that LPS is completely dissolved by the detergent is not true, then N_{LPS} will at least give a lower limit for the number of LPS monomers per micelle.

4. Results and discussion

4.1. Titration of a solution of aggregates with a fluorescent probe

The titration curves expected for the number of particles N in the confocal observation volume are depicted in Fig. 2 for

Fig. 2. Calculations of the titration curves from Eqs. (8) and (19). On the top is a sketch of the micelle labeling when going from low to high label concentrations. The curves represent the number of particles per observation volume as measured by FCS vs. the concentration of probe labels and are given for different values of the parameter $c_{\text{mic}} = (c_{\text{det}} - c_{\text{cmc}})/N_{\text{agg}}$ which describes the concentration of micelles. (A) Model assuming that all particles have the same fluorescent yield independent of the number of labels attached or that the concentration of labels is much smaller than the concentrations of micelles ($c_{\text{pr}} < 0.1 c_{\text{mic}}$, see Eq. (8)). (B) Model assuming that the fluorescence yield of a particle is directly proportional to the number of attached labels (Eq. (19), $\alpha_k \sim k$). (C) Model assuming that the fluorescence gets progressively quenched for every additional fluorophore incorporated into a micelle. Here progressive quenching of 50% ($\gamma = 0.5$, $\alpha_k \sim 0.5^{k-1}$) is assumed.



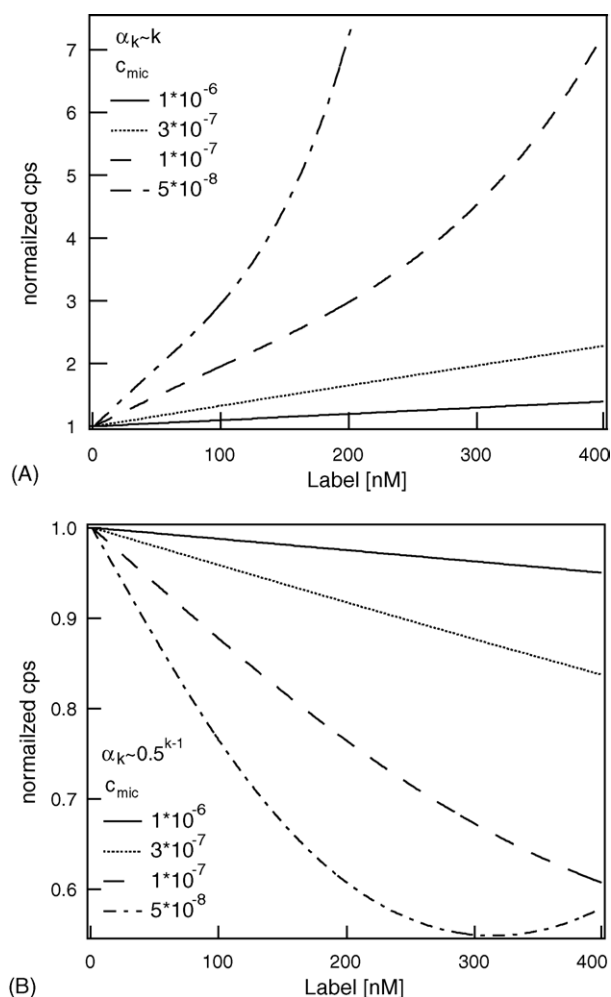


Fig. 3. Calculations of the titration curves from Eqs. (8) and (19). Depicted are the counts per particle and second (cps) as measured by FCS vs. the concentration of probe labels. The curves are given for different values of the parameter $c_{mic} = (c_{det} - c_{cmc})/N_{agg}$ which describes the concentration of micelles. The calculations are done for (A) the model assuming that the fluorescence yield of a particle is directly proportional to the number of attached labels as shown in Fig. 2B and (B) assuming progressive quenching with increasing label incorporation as shown in Fig. 2C.

three extreme cases. Firstly, all micelles have the same fluorescence yield $Q_k = Q_1$ (all $\alpha_k = 1$), independent of the number of labels contained. In this case the cps is constant during the titration. This model gives one limit of the values expected. From Fig. 2A it can be seen that during a titration, initially, N rises linearly with the label concentration as every particle incorporates one label ($c_{pr} \ll c_{mic}$). For higher label concentrations, when c_{pr} nears c_{mic} the probability that a particle contains more than one label becomes significant and the increase in N slows down and ultimately at very high concentrations ($c_{pr} \gg c_{mic}$) converges to the number of micelles present.

Secondly, we use a model in which the fluorescence yields per particle are proportional to the number of labels $Q_k = kQ_1$ (i.e. $\alpha_k = k$). For this second model we have depicted the cps in Fig. 3A. In this case N (Fig. 2B) also rises linearly with the label concentration ($c_{pr} \ll c_{mic}$) and this rise slows down when c_{pr} nears c_{mic} . However, for high label concentrations ($c_{pr} \gg c_{mic}$)

N decreases again. This is due to the fact that the FCS amplitude is proportional to the square of the fluorescence yield of the particles measured. Thus particles with a higher fluorescence yield contribute disproportionately more to the ACF than would be expected from their mole fraction (Eq. (15)). This leads to an increase in the ACF and thus a decrease in N . The cps in this case rises continuously since the average number of labels per particle increases continuously during the titration (Eqs. (3) and (4)) and the real number of particles is underestimated by the amplitude of the correlation function.

Thirdly, we use a model in which we assume that the fluorescence gets progressively quenched for every additional fluorophore incorporated into a micelle. The fluorescence yields per particle are then proportional to the quenching constant γ to the power of the number of labels minus one, since quenching happens only for $k > 1$: $Q_k = \gamma^{k-1}Q_1$ (i.e. $\alpha_k = \gamma^{k-1}$). In this case N (Fig. 2C) is dominated by singly labeled, unquenched particles and the normalized cps (Fig. 3B) decreases due to quenching. Again the real number of particles is underestimated by the amplitude of the correlation function.

In general, it can be seen that for concentrations of micelles larger than the concentration of labels, the different models have only a small influence on the number of particles N , since this parameter is dominated by the almost exclusively singly labeled micelles. In this situation differences between the three models can only be made by the normalized cps which will stay constant, increase or decrease, respectively. If the concentration of micelles is smaller than the concentration of labels, then N will be dominated by the multiple labeled micelles in the case of increasing fluorescence yield, or will be dominated by the singly labeled micelles in the case of quenching.

4.2. Determination of aggregation number of $C_{12}E_9$ using R18

R18 is a lipophilic fluorophore which is extensively used for membrane staining. Due to the long hydrophobic alkyl chain (Fig. 1A) R18 can be incorporated into the highly hydrophobic core of micelles. R18 in aqueous solution forms strongly quenched multimers [20] and exhibits ACFs with low N , low cps and at least two different diffusion times, consistent with aggregation and quenching. It exhibits high fluorescence only upon incorporation into hydrophobic structures and thus is a good probe for the detection of these structures, including micelles.

As a test case, we have studied the detergent $C_{12}E_9$ with a known CMC of 0.08 mM and N_{agg} of about 120 [13]. First, we tested the transition around the CMC by titrating fixed concentrations of 20 and 40 nM of R18 with $C_{12}E_9$ concentrations between 40 and 200 μ M and taking FCS measurements. In Fig. 4 the parameters N , cps and τ_D of the FCS fits are shown. All three parameters show a transition in the solutions between 70 and 120 μ M. The number of particles N (Fig. 3A) rises because R18, which is usually found in strongly quenched aggregates in solution, is dissolved in the micelles that are formed at concentrations of detergent higher than the CMC. With a known aggregation number of 120, 2.4 μ M of $C_{12}E_9$ will form 20 nM of micelles, enough to dissolve all R18 molecules. Thus the broad transi-

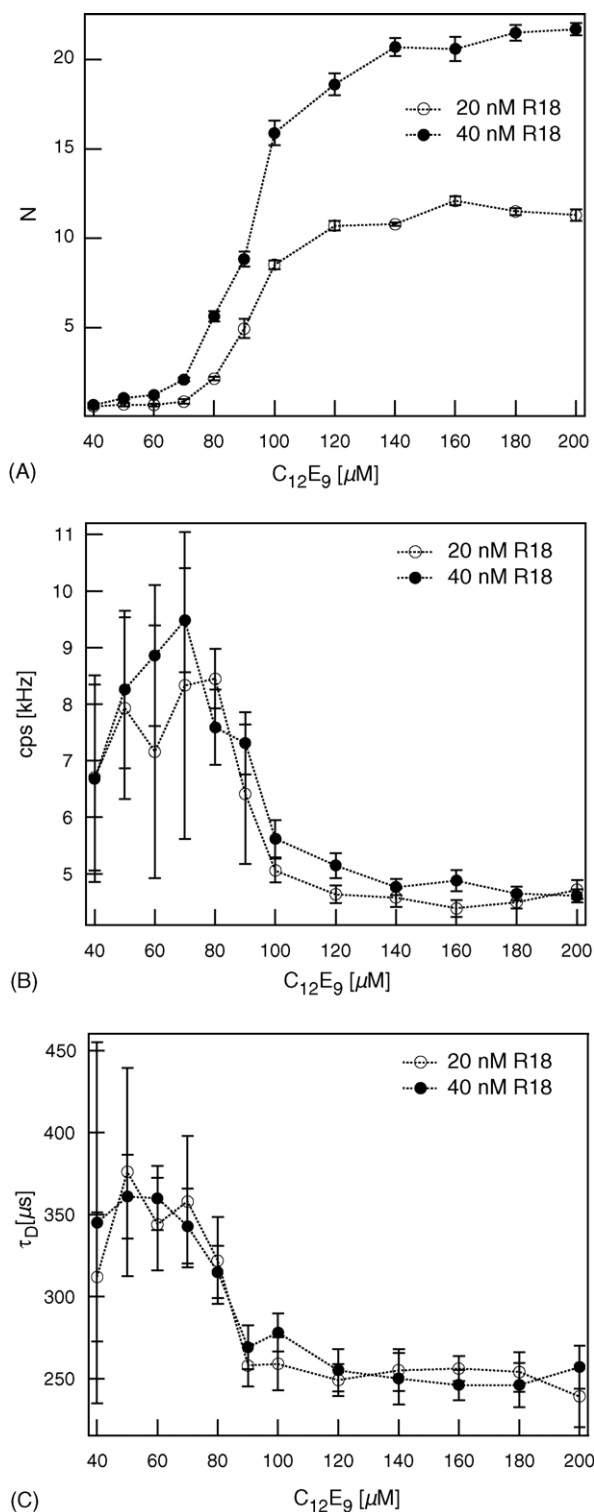


Fig. 4. Titration of 20 and 40 nM of R18 with increasing concentrations of $C_{12}E_9$ (40–200 μM). At least 10 FCS measurements were taken and the average fitted parameters are shown. (A) Number of particles N in the focal volume. (B) Counts per particle and second cps. (C) The diffusion time τ_D .

tion over more than 40 μM is not an artifact of the R18 label. This is confirmed by the fact that the transition is independent of the concentration of R18, and 20 and 40 nM R18 show the same transition. Thus the general assumption that the transition

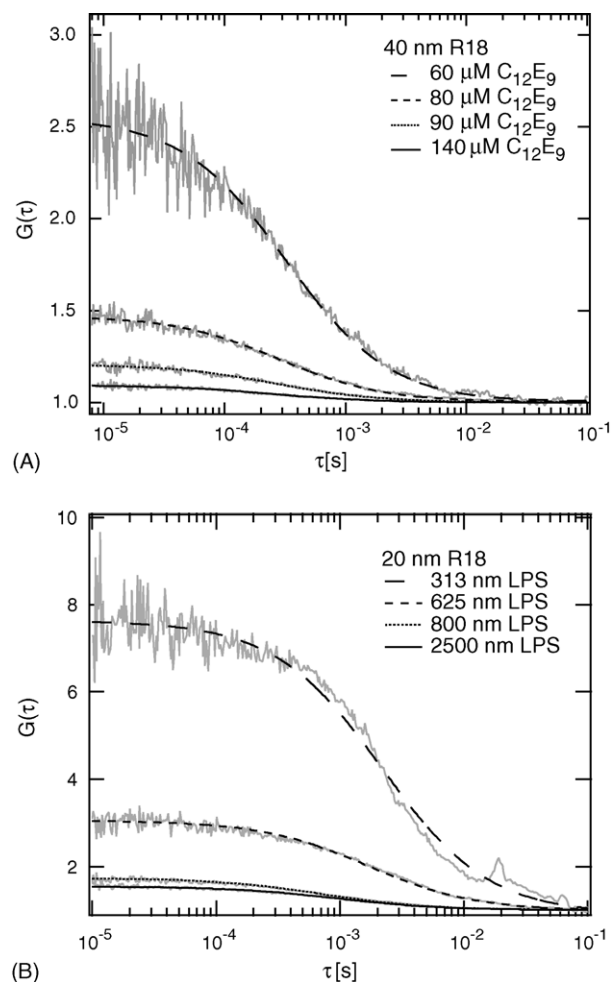


Fig. 5. Autocorrelation functions (ACF) taken in the transition region around the CMC (in grey). Fits assuming a single particle in solution are shown as dashed and solid black lines. (A) 40 nM R18 with varying concentrations of $C_{12}E_9$. (B) 20 nM R18 with varying concentrations of LPS. LPS shows especially at lower concentrations some aggregates as shown on the example of a concentration of 313 nm LPS.

at the CMC from monomers to micelles is abrupt is an idealization. The other two parameters, cps and τ_D (Fig. 4B and C) show a similar transition. Below a concentration of 90–100 μM these parameters fluctuate and show very large errors. Above this concentration range the parameters stabilize and show much smaller standard deviations, consistent with the idea that in this concentration range micelles dominate. Sample ACFs covering this transition are shown in Fig. 5A.

We have chosen 150 μM $C_{12}E_9$, a concentration above the CMC and the transition seen in our experiments, to test the aggregation number by titrating this solution with R18 concentrations from 0 to 450 nM. FCS curves are taken and the number of particles N and the cps is shown in Fig. 6A and B. In these measurements the cps increases slowly but steadily with R18 concentration indicating that more than one R18 label per micelle is incorporated on average. The models assuming either a constant fluorescence yield (Eq. (8)) or a fluorescence yield proportional to the number of labels (Eq. (19) with $\alpha_k \sim k$) are used to fit the data as limiting cases. The third model assuming

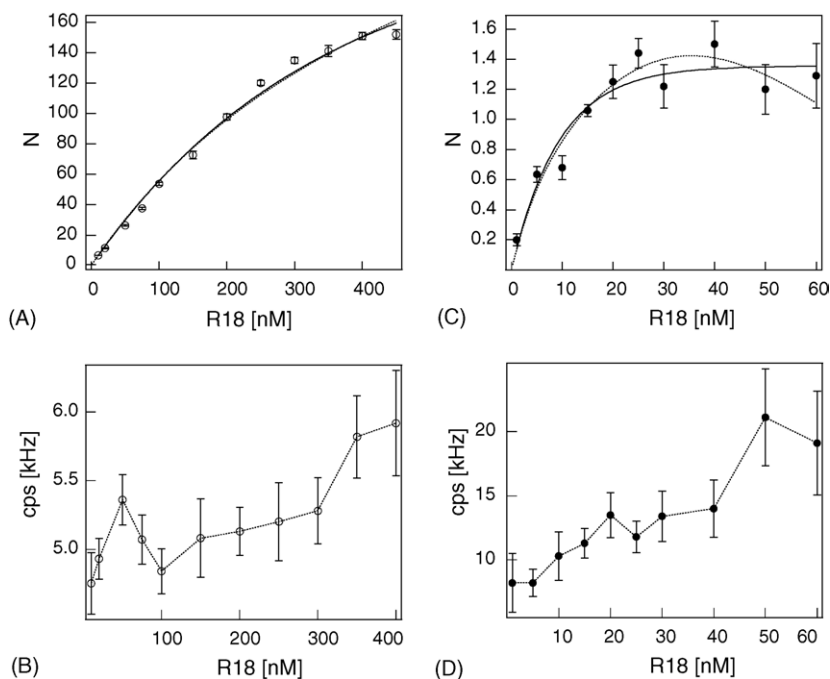


Fig. 6. Titration of $C_{12}E_9$ and LPS solutions with R18. At least 10 FCS measurements were taken and the average fitted parameters are shown. (A) The number of particles in the focal volume N for solutions of 0.15 mM $C_{12}E_9$ are shown for concentrations of R18 (10–450 nM). The dashed line is a global fit assuming that the fluorescence yield of a particle is directly proportional to the number of attached labels (CMC = 93 μ M, $N_{\text{agg}} = 112$). The solid line represents the result of a global fit to the model assuming that all particles have the same fluorescent yield independent of the number of labels attached (CMC = 105 μ M, $N_{\text{agg}} = 132$). (B) The counts per particle and second cps are shown for the same experiment. (C) The number of particles in the focal volume N for solutions of 2 μ M LPS are shown for concentrations of R18 (1–60 nM). The dashed line is a global fit assuming that the fluorescence yield of a particle is directly proportional to the number of attached labels (CMC = 1.6 μ M, $N_{\text{agg}} = 43$). The solid line represents the result of a global fit to the model assuming that all particles have the same fluorescent yield independent of the number of labels attached (CMC = 1.3 μ M, $N_{\text{agg}} = 49$). (D) The counts per particle and second cps are shown for the same experiment.

quenching and a decreasing cps is not applicable. Assuming a constant fluorescence yield the fit yields a CMC of 105 μ M and an N_{agg} of 132. Assuming a fluorescence yield proportional to the number of labels the fit yields a CMC of 93 μ M and an N_{agg} of 112. This is in good agreement with the theoretical values of a CMC of 80 μ M and an N_{agg} of 120 indicating that this titration method can be used to determine not only N_{agg} but as well the CMC. In all $C_{12}E_9$ experiments the observation volume is fitted to $0.9\text{--}1 \times 10^{-15}$ L.

4.3. Determination of aggregation number of LPS using R18

Fixed concentrations of 10 and 20 nM R18 were titrated with LPS concentrations from 0.25–5 μ M. In Fig. 7 the parameters N , cps and τ_D of the FCS fits are shown. A rise in N can be seen, stemming from the progressive integration of R18 into micelles. However, the transition happens over a much smaller concentration range of about 1 μ M. Experimental ACFs for this transition region are shown in Fig. 5B. The LPS solutions show especially at lower concentrations aggregates and in general the experimental values show higher errors than for the case of $C_{12}E_9$ (see Fig. 6). This indicates that the LPS solutions are less homogeneous and the aggregates have a wider distribution of sizes.

The results of the FCS measurements of 2 μ M LPS with increasing concentrations of R18 (0–60 nM) are shown in Fig. 6. In Fig. 6C the rise in the number of particles N with concentra-

tion of R18 can be seen as well as the saturation of this value for large R18 concentrations when all LPS micelles are labeled. Concomitantly, the cps (Fig. 6D) rises due to the increase in the average number of labels per LPS micelles. The data was fitted with the models of constant fluorescence yield (Eq. (8)) and the model of fluorescence yield proportional to the number of labels (Eq. (19) with $\alpha_k \sim k$). Assuming a constant fluorescence yield the fit yields a CMC of 1.3 μ M and an N_{agg} of 49. Assuming a fluorescence yield proportional to the number of labels the fit yields a CMC of 1.6 μ M and an N_{agg} of 43. In all LPS experiments the observation volume is fitted to $0.2\text{--}0.3 \times 10^{-15}$ L. This finding is consistent with the work of Santos et al. [9] who showed that LPS micelles change in aggregation number with concentration. However, the aggregate size we find for this LPS (*E. coli* 0111:B4) is larger than what was reported by Santos et al. for LPS (*E. coli* 026:B6). To find out whether there is a further change of LPS micelle size above 1 μ M we performed experiments on FITC-LPS micelles as explained in the next section.

4.4. Determination of aggregation number of LPS using FITC-LPS and Triton-X100

In this experiment, varying concentrations of FITC-LPS were dissolved by Triton-X100 (Table 1, Fig. 8). From 1 to 50 μ M FITC-LPS, the number of fluorescent particles N detected in the confocal volume increased linearly from 4.19 to 203.50, respec-

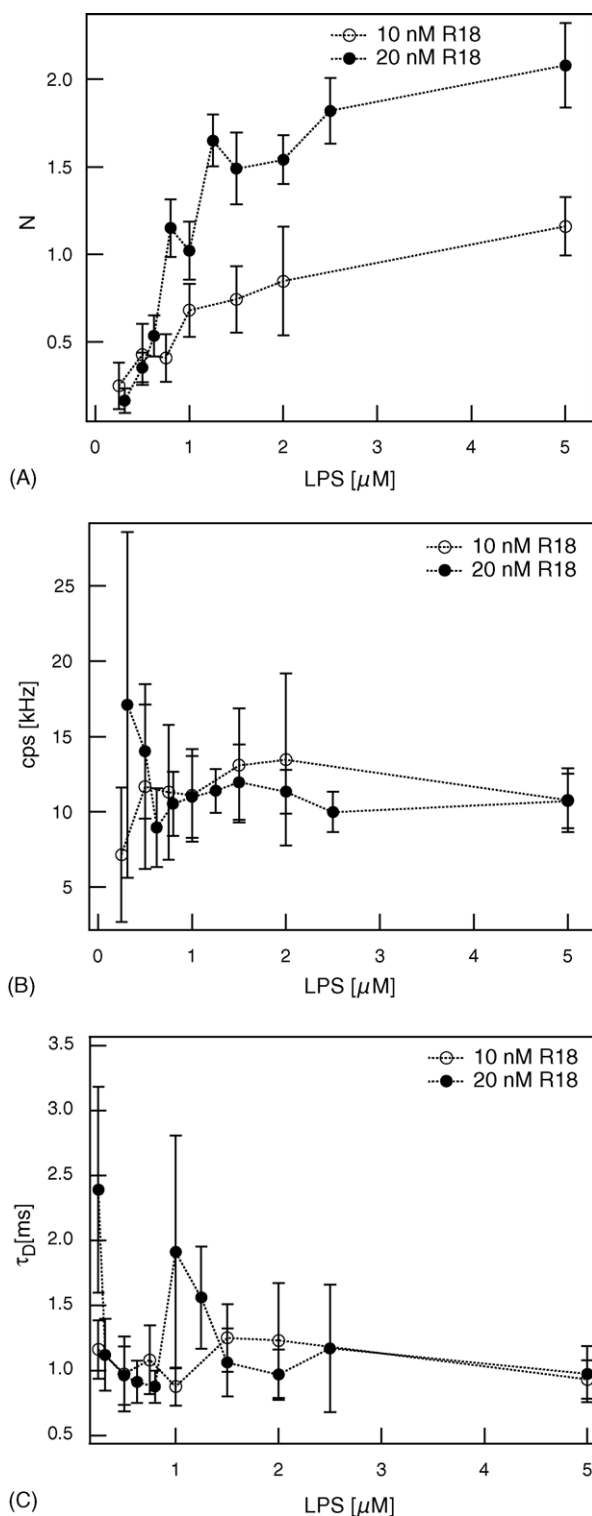


Fig. 7. Titration of 10 and 20 nM of R18 with increasing concentrations of LPS (0.25–5 μM). At least 10 FCS measurements were taken and the average fitted parameters are shown. (A) Number of particles N in the focal volume. (B) Counts per particle and second cps. (C) The diffusion time τ_D .

tively. Upon addition of Triton-X100, N increased by a factor between 1.67 and 2.26. Variations in this factor were not systematic and on average N increases by 1.94 ± 0.21 . In Fig. 8 these values are shown including linear fits to the data (FITC-LPS:

Table 1

Number N of particles per observation volume of FITC-LPS solutions of concentrations between 1 and 50 μM before and after treatment with Triton-X100 (17 mM)

FITC-LPS [μM]	N	N'	N'/N
1	4.2 ± 0.35	7.2 ± 0.41	1.72 ± 0.239
5	15.2 ± 1.94	34.5 ± 5.86	2.26 ± 0.673
10	40.2 ± 3.36	84.1 ± 7.58	2.09 ± 0.364
15	59.8 ± 1.82	123.4 ± 23.36	2.06 ± 0.454
20	71.1 ± 3.31	134.1 ± 16.72	1.89 ± 0.323
30	92.6 ± 8.19	154.6 ± 11.69	1.67 ± 0.274
50	203.5 ± 18.05	379.1 ± 31.01	1.86 ± 0.318

Values are given as mean \pm S.D. of 10 measurements. The last column shows the factor by which N increased upon addition of Triton-X100.

$y = -1.35 + 3.84x$ and FITC-LPS in presence of Triton-X100: $y = -0.36 + 7.03x$), since N should rise linearly with concentration. The slopes of the two fitted lines have a ratio of 1.83, consistent with the values 1.94 ± 0.21 determined from the measurements. Therefore, we conclude that on average there were 1.94 ± 0.21 particles per LPS micelle.

According to Sigma, the degree of substitution is 2–10 μg FITC per mg LPS which corresponds to 4–20% labeled LPS. Based on the LPS molecular weight of 10 kD, the degree of substitution showed that at 20% substitution, one out of five LPS molecule was labeled whereas at the lower limit of substitution, 4%, 1 out of every 25 LPS molecules was labeled. Combined with the estimation that one LPS micelle was labeled with 1.94 FITC molecules on average, therefore, there could be 10 LPS molecules per micelle if the LPS has 20% substitution. If the LPS has 4% substitution, the micelles would possibly have 50 LPS molecules per micelle. These values are a lower limit for the LPS aggregation number since the estimation relies on the assumption that LPS is completely dissolved and each Triton-X100 micelle contains at most 1 FITC-LPS molecule. Hence from this experiment, the number of LPS molecules per micelle

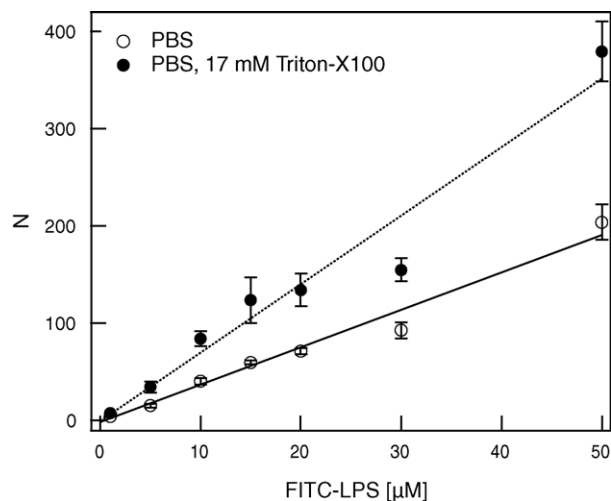


Fig. 8. Dissolution of FITC-LPS micelles by Triton-X100. The number of particles in the focal volume N , as measured by FCS, is shown vs. the concentration of FITC-LPS for solutions prepared in PBS buffer or in the presence of 17 mM Triton-X100. The data are fitted with a straight line.

is at least 10–50. While these results do not have a high precision because of the vague labeling conditions in which labeling efficiency is only given within a factor 5, they are consistent with the earlier measurements by FCS titrations showing that the LPS micelles contain 43–49 LPS monomers.

In general, the LPS solutions are less homogeneous than the C12E9 solutions and from time to time larger aggregates can be seen (Fig. 5). Therefore, the aggregation number is an average value around which the size of micelles will vary more or less strongly.

Santos et al. [9] have shown that LPS (*E. coli* 026:B6) micelle size varies at different concentrations. But their micelle sizes were smaller in aggregate size and transitions between small and large micelles happened at higher concentrations (10–20 μM). For LPS (*E. coli* 0111:B4) we found a change in aggregate size at concentrations of about 1 μM and could not detect any further changes in the aggregate size up to concentrations of 50 μM . This implies that temperature (Santos et al. work was performed at 37 °C) and the bacterial strain from which LPS is acquired significantly influence the solution behavior. Thus there is a need for a fast and easy method to measure the CMC and N_{agg} .

5. Conclusions

In this paper we have presented an alternative method to determine the critical micelle concentration (CMC) and the aggregation number (N_{agg}) of a molecular species by fluorescence correlation spectroscopy (FCS). By using the capability of FCS to measure concentrations of fluorescent particles we were able to determine the number of micelles, the CMC and the number of molecules per micelle N_{agg} by adding varying concentrations of a specific label to that solution.

The measurement of the mass of a particle by FCS is usually achieved by the determination of the diffusion coefficient (i.e. the width of the autocorrelation function [12]). This method is not universally applicable since the dependence of the diffusion coefficient on the mass is weak and thus prone to large errors. In addition the shape of the particle as well as the density of the particle has to be known, otherwise measurements are biased. The presented method which measures the number of molecules per micelle by using the amplitude of the correlation function, and thus the concentration of fluorescent particles, does not suffer from these problems as long as the concentration of the molecule under investigation is known.

The method was first validated by determining the CMC and N_{agg} of C₁₂E₉, a well-known detergent. Experiment and literature values were in good agreement. The method was then used to investigate the CMC and N_{agg} of a lipopolysaccharide (LPS) from *E. coli* strain 0111:B4. A CMC between 1.3 and 1.6 μM was found, and the N_{agg} is 43–49 molecules per micelle at a concentration of 2 μM of LPS. Above 1 μM up to 50 μM no further aggregation could be found.

The N_{agg} and CMC can thus be determined with this method and FCS is a good tool to elucidate the characteristics of LPS from different bacterial strains.

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